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HEPATOBILOPANCREATIC DISEASES
AND MULTITUMORAL SYNDROMES SECTION

*CORRELATION BETWEEN AGING AND TUMORIGENESIS:
THE ROLE OF GENETIC FACTORS
IN WERNER SYNDROME
AND RELATED SYNDROMES*

Simona Benoni

PhD student, XXII cycle

Supervisor: Prof. Francesco Cetta

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Ph.D. dissertation board

Prof. Francesco Cetta

Professor of Medical Surgery, University of Siena, Italy

Prof. Mario DeMarchi

Professor of Medical Genetics, University of Torino, Italy.

Prof. Francesca Gensini

Professor of Medical Genetics, University of Firenze, Italy.

Ph.D. thesis reviewer

Prof. Francesco Cetta

Professor of Medical Surgery, University of Siena, Italy

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INDEX:

Introduction.....	1
Aim of the study.....	3
1. Progeroid syndromes	4
2. Bloom syndrome (BS).....	7
3. RecQL4 helicase associated syndromes.....	9
<i>Rothmund-Thomson syndrome (RTS)</i>	
<i>RAPADILINO syndrome</i>	
<i>Baller – Gerold syndrome</i>	
4. Werner syndrome.....	13
5. Mouse models.....	28
6. Atypical Werner syndrome: mutation on LMNA gene.....	29
7. Clinical aspects of Werner syndrome on the patients.....	30
8. Roles of WRN protein in DNA repair, senescence and cell transformation.....	33
9. Previous attempt of genotype – phenotype correlation.....	35
10. Present study.....	38
<i>The literature review</i>	
<i>Results from two patients diagnosed and followed in Siena</i>	
<i>Results of the research among elderly patients at a geriatric institute in Milan</i>	
11. Alzheimer disease and its impact on the elderly people.....	57
Discussion.....	63
Conclusion.....	68

Introduction

Werner syndrome (WS) (MIM ID # 277700) is a pleiotropic disease of premature aging involving short stature, tught, atrophied, and/or ulcerated skin, a characteristic “birdlike” facies, and high, squeaky or hoarse voice, premature graying and thinning of the hair and early onset cataracts.

Additional common symptoms include diabetes mellitus, hypogonadism, osteoporosis, osteosclerosis of the digits, soft tissue calcification, premature atherosclerosis, malformed teeth, and flat feet, rare or multiple neoplasms (Epstein et al, 1996; and clinical and molecular description from the International Registry of Werner Syndrome, website: <http://www.pathology.washington.edu/research/werner/database/>).

Most patients carry biallelic mutations of *WRN* gene (MIM ID * 604611; also known as *REQL2*), because this pathology is transmitted in an autosomal recessive manner.

WRN is a member of the RecQ DNA helicase family. One of the main functions of these proteins is the surveillance of newly synthesized daughter strands of DNA for incorporating errors, and their repair (Ozgenc and Laob, 2005).

A possible explanation of WS symptoms is that in the presence of a reduced or completely absent expression of this helicase, DNA replication is not correct, but abnormal, leading to accumulate DNA errors until cell death for apoptosis and increased tissue turnover.

In fact, most of *WRN* mutations determine a truncated protein and even though the genetic tests now available are able to determine a correct diagnosis of WS, in about 20% of cases, no mutation in this gene is detected, indicating genetic heterogeneity for this disease (Chen et al 2003). It could be explained by the fact that present molecular tests do not investigate intron and large regions flanking *WRN* gene, or the possibility that other and unknown genes are implicated in the pathogenesis.

About 15% of the patients have shown to carry dominant missense mutations of *LMNA* gene, coding for the nuclear envelop proteins Lamin A and Lamin C. Dominant missense mutations of this gene are responsible for a Werner-like syndrome (or named “atypical Werner syndrome”), which tends to have a more severe course and is associated with cardiomiopathy more than patients with *WRN* mutations (Chen, 2003).

Most initial works on WS has been undertaken in Japan, where an exceptionally high incidence of Werner syndrome has been reported, probably due to an isolated area and to the usage of consanguineous marriage into the population.

Perhaps, this old custom has created founder *WRN* alleles, i.e. as c.3139-1G>C, p.G1047fsX1061 (or mutation 4), and c.1105C>T, p.Arg369X (mutation 6), respectively, the first and second more frequent mutations among WS subjects worldwide.

A recent review of mutations occurring in USA and Europe showed an increased incidence of C>T transition substitutions at nucleotide 1105 occurring in 24% of patients.

Subjects with *WRN* syndrome are supposed to have lower survival, namely because of premature atherosclerosis and/or diabetes, or cancer.

The mean age of death in the initial review by Epstein (1996), was 46 or 48 years, whereas in the most recent review by Huang (2006) a mean survival of 54 years has been reported.

In particular, Huang and collaborators reported that approximately 43% of their cumulative multicentric series of 99 capes had cancer and about 40% had atherosclerosis, whereas type 2 diabetes mellitus was present in 90%, even if reporting of these signs could be incomplete, and may be indicative of the variable penetrance and age-dependence of these pathological alterations associated with WS.

However, the diagnosis of Werner syndrome was made only on the basis of clinical signs, considering that, in these reports, information on genetic alterations was lacking.

Therefore, no definite genotype-phenotype correlation has been attempted for many years.

Two main drawbacks impair this task: 1) the limited number of subjects with both genetic mutations and type and site of cancer precisely reported; 2) the type of inherited (autosomal recessive), germ-line mutations found in the two alleles. In most of cases the mutations are same in both alleles.

Therefore genotype-phenotype correlation is even more difficult.

Inherited genetic syndromes, totally, multitumoral syndromes too, show great phenotypic variability. However, in the same instances, definite genotype-phenotype correlation have been established, between mutations located in a given genomic area (may be related to the different type of truncated protein) and a given phenotype (i.e. higher incidence of cancer in a given organ or tissue, or higher incidence of non-cancerous manifestations).

Aim of the study

About 20 different mutations in the *WRN* gene have been found, which determine early aging and which are also related with different types of cancers.

Tumor development depends on alterations in genes, especially in oncogenes, on molecular alterations genetically determined and/or environmental factors (like as air pollution, diet, etc.).

There are from one hand some transcription factors and/or molecules important for biological pathways of the human growth, which obviously take part in the tumorigenesis; on the other hand known oncogenes play an important role in non neoplastic processes, and conversely alterations in tumor suppressor genes are responsible for both multiple tumors and benign malformations.

There are also genes responsible of changes that include the acceleration of the growing and aging processes and also tumor development.

Furthermore, nutritional abnormalities (obesity and contrary prolonged fasting) can influence as well the increased presence as the protection against the tumors onset, together with a delay of a normal aging process. Finally there is the different susceptibility to cancer due to factors related to sex, of hormonal or not-hormonal kind.

From the foregoing, it is apparent that the appearance of tumors is closely related to the phenomenon of aging and is a complex phenomenon that results from the interaction between the factors mentioned above such as genetic predisposition to cancers and/or to early aging, nutritional and dietary factors (obesity or contrary body weight below normal for prolonged fasting) factors related to sex and to environment.

The aim of the present thesis has been to analyze in detail the interactions between some genetically determined diseases, characterized by precocious aging and typical skin changes and other alterations of various organs and districts, which are typically present in elderly people and the concomitant presence of neoplasms.

In particular we focused on the possible correlation between germ-line mutations in the causative gene of progeroid syndromes like as Rothmund-Thomson syndrome, Bloom syndrome, but most in particular Werner syndrome, and the presence or type of associated cancers.

Therefore, in this study there was applied one “comparative” method, which has been successfully used to uncover genotype-phenotype correlations in other inherited syndromes, to try to detect similar correlations in patients with Werner syndrome, namely in those with more frequent germ-line mutations.

This goal was performed by collecting all patients with WS available in Siena and Milan and complete genetic and oncologic information reported in the literature and pooling them with a personal series.

These data could be useful for both early diagnosis of cancer (and then better treatment) in subjects with at risk mutations, and for a better knowledge of the role of the altered *WRN* protein in cancer development.

1. Progeroid syndromes

Progeroid syndromes (PSs) are rare genetic disorders mimicking clinical and molecular features of aging. The interest in exploring age-related hereditary disorders is associated both with their severity, leading in many cases to life span shortening, and with the expectation that identifying causative genes could help understanding some of the mechanisms underlying the physiological aging.

Indeed, all the clinical and biological changes usually observed during natural aging are never totally recapitulated in PSs. To date, most of these diseases, for which genes and pathophysiological mechanisms have been identified, are monogenic and fall into the category of segmental PSs. In this context, Werner syndrome (WS) and Hutchinson–Gilford progeria syndrome (HGPS) have been the most extensively studied.

The syndromes of accelerated aging have been proposed as models to simplify the analysis of the normal aging process, by restricting the focus to a more definable area.

Research in this field may give an insight into the nature of the genes that play a role in aging and help to separate the boundary between aging and age-related disease. Furthermore, the association of various cancers and vascular pathologies in many of these conditions has allowed a better understanding of the genetic or intrinsic components of these important causes of premature death.

Some of all different progeroid syndromes are due to helicase proteins-linked disorders like as Werner syndrome, which is of our particular interest in this thesis.

RecQ helicase family

Helicases are the enzymes that separate the complementary strands of energetically stable, double-stranded, nucleic acid structures utilizing the energy derived from hydrolysis of ATP. RecQ helicases proteins act on DNA substrates, and may play a role in the replication of specific DNA sequences or in the generation or resolution of DNA recombination, or repair events that require at least a limited degree of DNA synthesis (Fry and Loeb, 1998). The single-stranded (ss) RNA or DNA molecules thus produced are then used as templates or substrates in various biological processes, such as DNA replication, repair and transcription (Hanada K, Hickson ID 2007).

In elementary organisms too, like as bacteria and yeast, RecQ helicase are more important for the genetic stability assurance, besides other previously indicated functions.

Interestingly, the number of RecQ enzymes expressed by a particular organism is apparently correlated with their genome size.

Mammals and birds have five homologues helicase proteins (Fig 1); in humans they are designated RECQ1, BLM (responsible for the Bloom syndrome), WRN (Werner syndrome), RECQL4 (Rothmund-Thomson syndrome) and RECQL5 (Ellis NA et al 1995; Yu CE et al 1996, Kitao S et al 1998).

WRN, RECQL4 and BLM helicases are relatively well studied, because defects of these proteins cause human premature aging-linked pathologies and cancer predisposition; but comparatively little is known about human RECQL1 and RECQL5, and it is not yet clear whether the five human RecQ homologs provide partially redundant, complementary or independent cellular functions.

To date, no human pathologies have been found with deficiencies in either RecQL1 or RecQL5.

It could be simple to retain that these progeroid conditions are all very similar each other, but it is not true, because while some of the clinical features of each RecQ associated diseases overlap, some are unique, suggesting that the RecQ helicases may have differential cellular roles that translate into the distinct symptoms (Rossi et al. 2010).

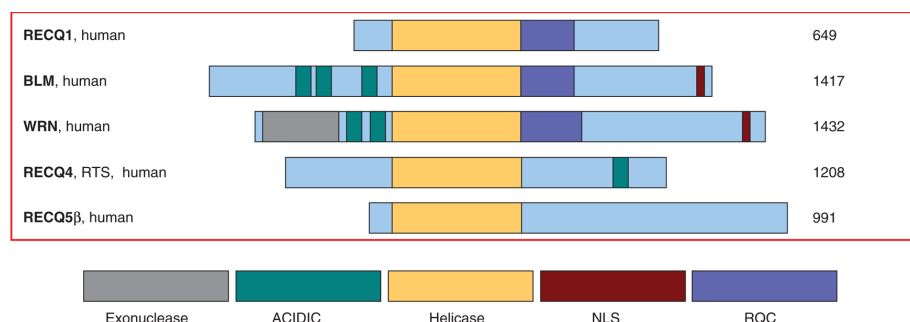


Fig 1: Schematic diagram of the 5 RecQ helicases proteins.

Conserved domains are aligned and color-coded, as indicated at the bottom of the figure. The size of each protein species in amino acids is indicated on the right. RECQ5 exists in three isoforms, the human β form is shown (Bohr 2008).

RecQ helicases functions

DNA damage is mediated by a complex network of DNA-repair proteins, many of which form multifunctional multiprotein complexes that participate in more than one DNA-repair pathway (Hoeijmakers 2001).

Is demonstrated that RecQ helicases can interact with DNA-repair other proteins and it may co-participate in specific DNA-repair processes. This is consistent with the observation that RecQ helicases have structure-specific DNA binding sites and have higher affinity for DNA substrates, that resemble DNA-repair intermediates than for simple duplex DNA (Mohaghegh et al. 2001; Opresko et al. 2004a), and it was shown that different other proteins are able to link with them, in particular proteins belonging to the four main DNA-repair systems (Fig. 2):

- base excision repair (BER), which repairs oxidative DNA base modifications such as 8-oxoguanine (8-oxoG), alkylation base damage and ssDNA breaks (SSBs);
- nucleotide excision repair (NER), which repairs bulky helix-distorting DNA lesions;
- mismatch repair (MMR), which repairs single-nucleotide mismatches and small insertion–deletion mispairs;
- DSBR, which repairs double strands breaks.

The ubiquity of helicases reflects their important roles in virtually all aspects of nucleic acid metabolism.

We report here a small description of human RecQ helicases functions and pathological dysfunctions:

RecQL1: DNA helicases involved in various types of DNA repair, including mismatch repair, nucleotide excision repair, and direct repair.

RecQL3 (BLM): enzyme associated with an autosomal recessive disorder, characterized by severe pre- and postnatal growth retardation. The rash in Bloom syndrome is characterized by neonatal blistering, distinctive facial erythema, and telangiectases and develops in sun-exposed areas of the skin; it is not a true poikiloderma. Individuals with Bloom syndrome may also have café-au-lait spots or paired hypopigmented and hyperpigmented spots. Recurrent infections (otitis media and pneumonia), chronic pulmonary disease, and diabetes mellitus are common. Many have learning disabilities. The most common cause of death is cancer (epithelial, hematopoietic, lymphoid, connective tissue, germ cell, nervous system, or kidney), which occurs at younger than usual ages.

RecQL2 (WRN): helicase responsible for Werner syndrome. Individuals with this disorder are characterized by the premature appearance of typical features associated with normal aging and by cancer predisposition. There are observed loss and graying of hair, alopecia, hoarseness, and scleroderma-like skin changes, followed by bilateral ocular cataracts, type 2 diabetes mellitus, hypogonadism, skin ulcers, and osteoporosis in the 30s. Myocardial infarction and cancer are the most common causes of death.

RecQL4: defects of this protein are associated with Rothmund-Thomson syndrome (RTS) through an autosomal recessive inheritance. In affected individuals they determine the presence of sparse hair, sparse eyebrows/lashes, small stature, skeletal abnormalities, cataracts, and an increased risk of cancer, especially osteosarcoma. The skin typically is normal at birth; but skin lesion develops between age three and six months as erythema, swelling, and blistering on the face, and subsequently it spreads to the buttocks and extremities. Over months to years, the rash evolves into a chronic pattern of reticulated hypo- and hyperpigmentation, punctuate atrophy, and telangiectases, collectively known as poikiloderma.

RecQL5: helicase protein with 7 consensus motifs in the middle of the molecules. Its expression in almost all tissues tested, with notably strong expression in pancreas and testis. The functions of RecQL5 are so similar to those of BLM, that it was proposed that RecQL5 might possess a role in the control of Sister Chromatid Exchange (SCE) levels that is redundant to that of BLM (Hanada K and Hichkson ID, 2007).

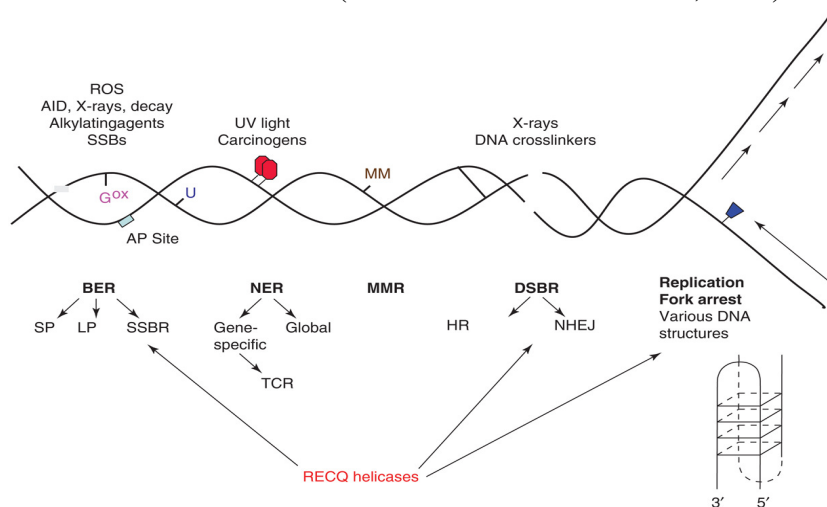


Fig 2: Eukaryotic DNA repair. Schematic diagram summarizing eukaryotic DNA-repair pathways and their target DNA lesions (Bohr, 2008).

2. Bloom Syndrome (BS)

Clinical features and molecular genetics of BS

Bloom syndrome (BS) is a rare autosomal recessive disease characterised by severe growth retardation and dramatic cancer predisposition (Bloom D 1954, German J 1993).

The most common physical characteristics of affected individuals are congenital short stature, a narrow face with a prominent nose, a characteristic butterfly-shaped facial rash that is induced by sunlight exposure, a high-pitched voice and abnormal skin pigmentation, especially on sun-exposed parts of the body (Fig. 3). Some BS patients also show learning disability, mental retardation, immune deficiency, diabetes and/or mild anaemia.

Men with BS cannot produce mature sperm and this results in infertility. Affected females are rarely infertile (Chisholm CA et al. 2001).

Fig 3: A representative example of Bloom syndrome aspect. Evident is the abnormal skin pigmentation on the face.



There is an elevated risk of developing common adult epithelial tumors such as colon, breast and lung cancer; leukaemias and lymphomas. With the possible exception of melanoma (perhaps because affected individuals avoid sunlight exposure) it would appear that BS individuals succumb to the full range of cancers seen in the normal population.

However, they occur so several decades earlier in life than is expected. Some patients also develop tumors that are normally very rare in the general population, such as osteosarcoma, Wilms tumor, and medulloblastoma (Hanada K and Hickson ID, 2007).

Genomic instability is proposed to drive tumorigenesis in BS. Significantly, a higher frequency of somatic mutation is observed in cells from BS patients, besides an abnormally high rate of micronucleus formation and homologous recombination (Traverso G et al. 2003), and an increased rate (10-fold) of sister-chromatid exchanges (SCEs), which is used as a molecular diagnosis of this disorder.

Bloom syndrome is extremely rare, but is somewhat more common in Jewish persons of Eastern European descent (Ashkenazi Jews) (German J et al, 1977).

Biochemical functions of BLM

BLM (encoded by *RECQL2* gene, located at 15q26.1) is a helicase that separates the complementary strands of duplex DNA.

BLM can unwind 3'-tailed duplexes, bubble structures, forked duplexes, G-quadruplex structures, DNA displacement loops (D-loops) and four-way junctions modelling the Holliday junction recombination intermediate (Bachrati CZ et al, 2006).

Interestingly, BLM possesses what appears to be the opposite activity of a helicase. BLM can catalyze the annealing of the complementary single strands of DNA (Cheek C et al, 2005). The annealing activity of BLM, unlikely its helicase activity, is inhibited by ATP. This suggests that ATP binding and hydrolysis triggers an alteration in the mode of action of BLM.

Biochemical studies showed that BLM preferentially disrupts two structures that typically form as intermediates in homologous recombination; D-loops and Holliday junctions (Bachrati CZ et al, 2006; van Brabant et al, 2000), named above.

One possible role for BLM is to negatively control homologous recombination reactions, perhaps by unwinding the invading strand from the D-loop. If the D-loops are converted into Holliday junctions, these junctions have to be resolved otherwise, the recombining molecules will remain covalently intertwined.

There are three possible explanations for BLM action to prevent wrong recombination events.

First, BLM might suppress crossovers by destroying all forms of aberrant or unwanted strand invasion events through D-loop unwinding.

Another possibility is that BLM promotes the process of synthesis-dependent strand annealing (SDSA) that only gives raise to non-crossovers.

A third possibility is that BLM promotes resolution of Holliday junctions to generate exclusively or predominantly non-crossover products.

However, there are data indicating that BLM localises to anaphase bridges that represent delayed or failed sister-chromatid disjunction, suggesting a role for BLM directly in “resolving” such structures.

BLM mutations

Most of the mutations in BS patients are either nonsense or frameshift mutations that cause the truncation of the protein product; they are distributed on all the length of the BLM protein, with a particular concentration on the C-terminal region, characterized by the presence of catalytic domains (Fig. 4).

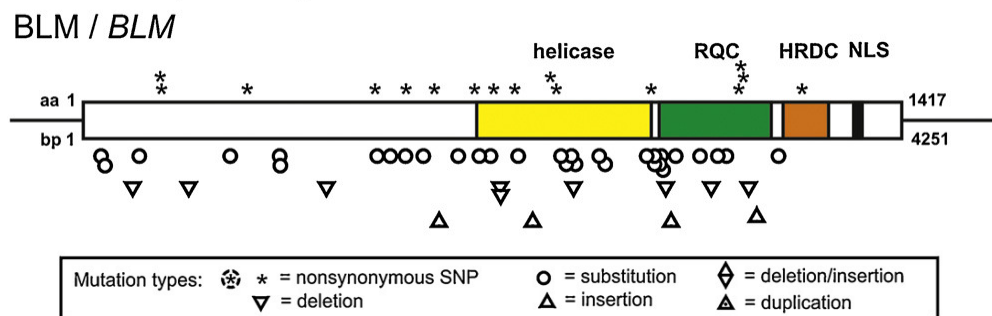


Fig. 4: Disease-causing mutations in human BLM gene (Monnat RJ et al. 2010)

These truncated BLM proteins are expected to be non-functional, because they either lack the essential catalytic helicase domain and/or the nuclear localization signal (NLS), located in the C-terminal region of BLM (Hayakawa S et al, 2000), though not in all cases eliminate expression of the mutant protein.

This suggests that BLM helicase activity is the major determinant of BLM-associated phenotypes in human somatic cells, regardless of whether the mutant protein is lost or not.

3. RecQL4 helicase associated syndromes

Rothmund -Thomson syndrome (RTS)

RTS is an autosomal recessive genodermatosis characterized by poikiloderma, graying and loss of hair at an early age, juvenile and bilateral cataracts, short stature, skeletal and dental abnormalities, radial ray defects and a predisposition to cancer (especially osteosarcoma).

RTS is a very rare disease and reliable data on its prevalence are not available.

To date, approximately 300 patients have been recorded in the medical literature (Vennos et al. 1992; Wang LL 2006).

Teeth, like as hair and nails are epithelial appendages and their malformations such as microdontia, rudimentary or hypoplastic teeth, multiple and unusual crown formations, are classified as typical cutaneous rather than as extracutaneous manifestations of the disease (Roinioti 2007). Growth delay and the resulting characteristic short stature, however proportionate and without asymmetry, is the second major clinical sign of RTS.

The skin is usually normal at birth, but red patches start to appear at between 3 and 6 months of age. Subsequently, poikiloderma, that is the mainly characteristic of the disease, develops and persists throughout life (Fig. 5).



Fig 5: Panel showing some clinical features of the RTS syndrome.

A-B-C) Chronic phase of cheek poikiloderma

D) Poikiloderma sparing the trunk

E) Photo distributed poikiloderma and valgism of the knees.

F) Thumb aplasia (B).

G) Bone defect seen by X-Rays: cystic-like destructive lesion of the humerus without apparent solution of continuity of the cortical bone (E).

Radiograph shows aplasia of right thumb (right bottom)

Some patients show photosensitivity, although this is highly variable. But it is notable that any skin cancer that develops in RTS cases can occur on skin regions not normally exposed to the sun.

Neurologic cognitive milestones and intelligence are usually normal, although are described cases with mental retardation, may be induced by cerebral atrophy.

About 68-75% of the patients were found to display skeletal anomalies, including frontal bossing, saddle nose and abnormalities of the long bones. The most common anomalies were abnormal metaphyseal trabeculation, brachymesophalangy, thumb and/or radial aplasia or hypoplasia, osteopaenia, and patellar ossification defects (Larizza L et al. 2010). Whether RTS has a predilection for one sex over the other is unclear. In fact, both a female predominance (1.4:1), and a male predominance (2:1) have been reported in various case series.

This disorder can be divided in 2 sub-categories.

Type I RTS, characterized by poikiloderma and juvenile cataracts; not caused by RecQ family genes mutations, is an orphan Mendelian disease, for which the responsible gene/s is/are not yet found; and type II RTS, characterized by poikiloderma and skeletal defects, is caused by homozygous or compound heterozygous mutations in the *RECQL4* gene.

For this reason, *RECQL4* is the only gene associated with RTS to date, and although evidence suggests genetic heterogeneity for a not indifferent part of patients (34% of the entirely RTS cases), no other locus for RTS has been identified.

RecQL4 protein functions and activities

The enzymatic function of RecQL4 (encoded by *RECQL4* gene, located at 8q24.3) is poorly understood, however is known that it plays a role in a DNA-dependent ATPase activity and in single-stranded DNA annealing activity (that is similar to what seen in the others RecQ helicases); but its helicase property is doubtful and RecQL4 appears to be distinct from all other members of the RecQ helicase family (Macris et al. 2006).

Localization of the protein was shown to be both nuclear and cytoplasmic: nuclear localization and retention domains are amino-terminal, unlike other RecQ proteins which show carboxyl-terminal nuclear localization signals (NLS) (Burks et al. 2007).

A cumulative set of data suggest that RecQL4 has roles in several different important cellular pathways: essential for correct chromosome segregation and apoptosis, for the DNA double strand breaks repair (DSBR), for transcriptional regulation and in the base excision repair (BER) (Kumata et al. 2007).

In fact, it was reported that RecQL4 plays a role in oxidative stress and its amount increases in the nucleolus after treatment of cells with agents that induce reactive oxygen species (ROS) (Woo LL et al. 2006), explaining its role effectively where there could be done a cellular damage. A defect in responding to ROS might explain the development of premature aging in RTS, as one of the hallmarks of aging is the accumulation of reactive oxygen species.

Moreover, RecQL4 protein is significantly expressed in the S-phase of the cell cycle, suggesting a role also in DNA replication, and in particular, in replication fork processing.

RTS and cancer onset, association and predisposition

Given the multiple roles of RecQL4 in DNA metabolism, it is likely that in RTS patients with *RECQL4* mutations defective DNA replication, enhanced oxidant sensitivity and that unrepaired DNA lesions would lead to sustained genomic instability.

In vivo, chromosomal instability may drive neoplastic transformation of cancer stem-cells. This may be true for cells of the mesenchymal compartment that give rise to the tumors most frequently developed by RTS patients.

As compared to chromosomal instability features of other RecQ helicase defects, RTS has apparently no overlap with Bloom's syndrome, because no increase in sister chromatid exchanges (SCEs), has been recorded in RTS cells (Grant et al. 2000, Lindor et al. 2000).

RTS could resemble Werner syndrome, with respect to a few clinical features and the increased predisposition to mesenchymal tumors.

However, the spectrum of chromosomal rearrangements appears more restricted in RTS than in Werner cells, where the "variegated translocation mosaicism" is enhanced by multiple pathways, including telomere erosion and chromosome end fusions (Ouyang et al. 2008).

Cancer predisposition is a central sign of the disease.

It is not surprising that the most frequent tumor types found in RTS patients, osteosarcoma (generally in childhood or adolescence) and spinocellular carcinoma of the skin (squamous cell carcinoma, basal cell carcinoma and Bowen's disease, in the adult age) develop in the cellular compartments most severely affected by the disease. Both types of cancer develop at an earlier age than would be normally expected.

Malignant haematological abnormalities ranging from myelodysplasia to aplastic anaemia and leukaemia have been identified (Rizzarri et al. 1996; Porter et al. 1999).

Numerous and various mutations are found in RECQL4 gene, especially at level of helicase or RCQ domains; and among these alterations there is an unusually high proportion of recurrent mutations that disrupt splicing.

This is likely explained by the unusual genomic structure of the human *RECQL4* gene, where 13 out of 20 exons are short (<100bp long) and prone to stochastic or mutation-induced mis-splicing.

A second mechanistically intriguing observation is mutational sparing of the N-terminal portion of RecQL4. In fact, the majority of mutated alleles identified thus far in RTS patients are predicted to encode absent or truncated proteins (Siitonen, Ha et al, 2003).

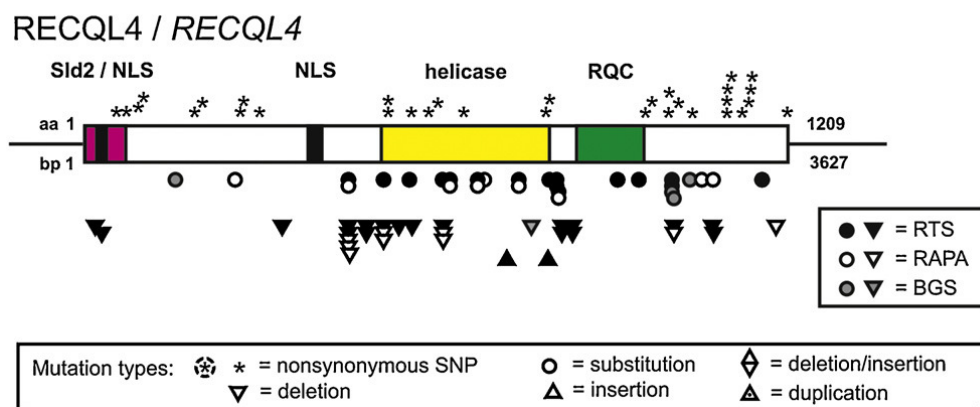


Fig 6: RECQL4 mutations; those identified in Rothmund–Thmonson, RAPADILINO or Baller–Gerold syndrome patients are further indicated by the symbol fill, with the key to the lower right (Monnat RJ et al 2010).

Genotype-phenotype analysis showed that all patients with the *RecQL4* mutation (and particularly, with a truncating mutation) had skeletal abnormalities and a related increased risk of cancer, especially osteosarcoma, which was thought to be determined by the presence of at least one truncating mutation (Wang LL et al. 2003).

RAPADILINO syndrome and Baller-Gerold syndrome (BGS):

Other two genetics conditions RAPADILINO syndrome and Baller-Gerold Syndrome (BGS) are also caused by the mutations in *RECQL4* gene (Siitonen, Ha et al, 2003; Van Maldergem L et al, 2006).

- RAPADILINO syndrome (a rare autosomal recessive disorder) is characterised by radial hypoplasia or aplasia (RA), patellar hypoplasia or aplasia and cleft or high arched palate (PA), diarrhoea and dislocated joints (DI), little size and limb malformation (LI), and nose slender and normal intelligence (NO).

People with RAPADILINO syndrome do not display obvious cancer predisposition.

The other distinct difference between RAPADILINO and RTS is the occurrence of poikiloderma that is one of the hallmarks of RTS, but is only rarely observed in patients with RAPADILINO syndrome.

- Clinical overlap between BGS, Rothmund-Thomson syndrome (RTS), and RAPADILINO syndrome is noticeable. In fact, BGS symptoms are radial ray hypoplasia, skeletal dysplasia, short stature, and craniosynostosis. Poikiloderma is also observed.

Van Maldergem (2006) re-evaluated 2 previously reported BGS families and found causal mutations in the *RECQL4* gene in both, confirming that BGS in a subgroup of patients is due to *RECQL4* mutations and belongs to a clinical spectrum that encompasses RTS and RAPADILINO syndrome.

However, palatal abnormalities and joint dislocation are only seen in RAPADILINO syndrome. Cataracts, dental and nail abnormalities, sparse hair, and cancer predisposition are typically observed only in RTS patients.

Although a range of different *RECQL4* mutations have been found in these syndromes, including nonsense mutations, missense mutations, frameshifts, splice site mutations, and intronic deletions, it is difficult to reconcile how these mutations lead to three distinct syndrome.

4. Werner syndrome

Clinical characteristics of the disease

The clinical phenotype of Werner syndrome (WS) is best summarized as early onset of an aged-appearance and age-related common disorders (Epstein et al. 1966; Goto 1997) or more simply a disease that “mimics” the normal aging. In fact, Werner syndrome is characterized by the premature appearance of features associated with normal aging and cancer predisposition (Fig. 7).

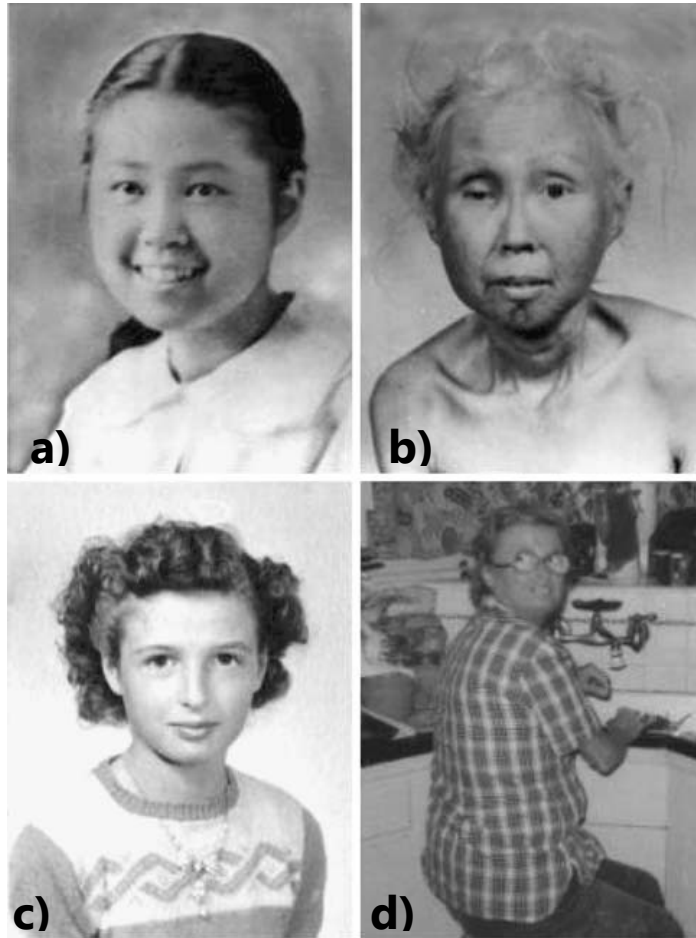


Fig. 7: Examples of Werner syndrome patients. Japanese-American WS patient at ages 15 (a) and 48 (b) was originally reported in Epstein et al. (1966) showed early graying and thinning of hair, skin atrophy, loss of cutaneous fat and general aged feature. A Caucasian WS patients, at age 8 (c) and 36 (d) developed bilateral cataracts, thinning of hair, atrophic skin and thin limbs (Hisama et al. 2006).

WS patients usually develop normally and therefore pathological characteristics are not apparent until these individuals reach the third decade of life.

Generally, the first clinical sign is a lack of the pubertal growth spurt during the teen years. Patients frequently recall that they were of average height when they entered grade school, but were the shortest ones in their class, when they graduated from high school. A clinical study of Japanese WS reported the median height of affected individuals was 142 cm (range 122–161 cm) which was 13 cm shorter than the general population (Goto 1997). Median body weight was 36 kg (range 19–52 kg) that was 20 kg less than the general population.

In their 20s, patients begin to manifest the first symptoms, like as skin atrophy or graying/loss of hair.

Subcutaneous fat tends to deposit on the trunk and combined with osteoporosis of the limbs, so patients exhibit a stocky appearance. Some individuals may present a high-pitched voice and fat feet.

Subsequently, they develop common age-related signs or disorders, such as hoarseness and scleroderma-like skin changes, followed by bilateral ocular cataracts, type 2 diabetes mellitus, hypogonadism, skin ulcers, and soft tissue calcification, premature atherosclerosis and osteoporosis; all these could be manifested in their 30 years.

A recent survey of WS patients with a molecularly confirmed diagnosis revealed that the prevalence of cataracts was 100% and also, the prevalence of osteoporosis at the time of diagnosis was 91%, hypogonadism 80%, diabetes mellitus 71%, neoplasms 43% and atherosclerosis 40% (Huang et al. 2006) (Tab. 1).

Sign or symptom	Percent frequency
Bilateral cataracts ^a	100 (87/87)
Skin alterations ^a	98.6 (72/73)
Thin limbs	98.4 (60/61)
Premature graying/hair loss ^a	96.3 (79/82)
Pinched facial features	96.1 (49/51)
Short stature ^a	94.7 (71/75)
Osteoporosis	90.6 (48/53)
Voice change	89.0 (65/73)
Hypogonadism	79.5 (35/44)
Type 2 diabetes mellitus	70.8 (46/65)
Soft tissue calcification	66.7 (28/42)
Neoplasm(s)	43.6 (24/55)
Atherosclerosis	39.5(17/43)
All four cardinal signs ^a	90.9 (50/55)

Tab. 1: Clinical features of Werner syndrome in subjects with molecular documentation of the diagnosis. (The number of the cases where information was available is given in the parentheses) (Huang et al. 2006).

This disease is generally rare, but it should be noted that most of the existing patients (845 of the 1200 cases reported worldwide, about two thirds of the total) are Japanese, and also have indicated that 1 in 160 of the Japanese population is a carrier of the disorder (Hanada K and Hickson, 2007). The chronological order of the onset of these complications doesn't change between the populations; in fact, it is similar among Caucasian and Japanese WS patients (Epstein et al. 1966; Goto 1997).

In particular, Japan is considered to be a unique model for the analysis of the genetic epidemiology of WS because it is geographically isolated with relatively few population exchanges, and represents the highest frequency of this disease in the world.

Median age of death was recently updated to 54 years (Huang et al. 2006, Rossi et al 2010). This represents a significant increase over what had been observed several decades ago, and is probably the result of improved medical management.

At this moment, there is no cure for WS, and the most common causes of patients' death include atherosclerotic vascular diseases, such as coronary heart disease, myocardial infarction, cerebral vascular diseases and malignancies (Epstein et al. 1966; Goto 1997).

Although the last two reasons are common causes of death also in the general population, most important is to understand which types of cancers are observed in WS patients.

In total, 8% of patients with WS develop malignant tumors, which are usually diagnosed in the second or third decade of life.

Types of cancers associated with Werner syndrome

Mesenchimal tumors (connective tissue)	Fibrosarcoma Osteosarcoma Malignant Fibrous Histiocytoma (MFH) Leukeamia Malignant soft tissue tumor Anaplastic nasal cancer Rhabdomyoblastoma Leiomyosarcoma Meningioma
Epithelial tumor (carcinomas)	Thyroid cancer (follicular and papillary) Breast cancer Squamous cell tumor of nose Squamous cell tumor of hands Bladder cancer Basal cell carcinoma Uterine cancer Cancer of the tongue Cholangioma Ovarian cancer Laryngeal cancer Gastric cancer Lung cancer
Other types	Malignant melanoma Glioma multiple myeloma

Tab. 2: Types of malignant tumors identified in a cohort of 85 WS patients according to the search of MEDLINE reported cases and Japanese databases, from 1916 to 2002.

The *spectrum* of neoplasms occurring in Werner syndrome is unusual, because it includes a large number of sarcomas and those types of cancer which are very rare in the general population (Goto et al 1996, Yamamoto et al 2003) (Tab. 2).

The most common tumors in Japanese WS individuals (for whom the most data exist) are soft-tissue sarcomas, thyroid carcinomas, melanomas, and osteosarcomas. Acral lentiginous melanomas (most often observed on the feet and nasal mucosa) are particularly prevalent compared to levels observed the general population and are unrelated to sun exposure (Goto et al. 1996).

So, the ratio of cancers of epithelial origin and sarcomas of mesenchymal origins is 1:1 in WS patients, whereas this ratio is approximately 10:1 in the general population (Goto et al. 1996).

The primary sites of osteosarcomas in WS patients are more likely to be in the lower extremities, whereas these are more common in the upper extremities in the general population (Ishikawa et al. 2000).

There are other differences that have been noted between WS patients and normal elders. Fertility, for example, appears to decline in both sexes soon after sexual maturity. This decline is associated with testicular atrophy and probable accelerated rate of loss of primordial follicles in the ovaries; although data are sparse (the cells isolated from WS patients show a reduced ability to proliferate, which may be one of the underlying reasons for premature aging). Early menopause is common in women as are multiple miscarriages, but successful pregnancies have also been reported. Men have fathered children, usually at younger ages (Epstein et al 1966), but after they became infertile.

Atherosclerosis exhibits unique characteristics in WS patients, since atherosclerotic lesions are more extensive in arterioles.

Skin ulcers around the ankles and elbows, that are far more severe than those expected because of the frequently associated diabetes mellitus, are common in WS. While in the

general population osteoporosis has a more pronounced effect on vertebrae, in WS patients, long bones (particularly those of the legs), tend to be more affected by osteoporosis (Epstein et al. 1966; Rubin et al. 1992), although more studies are needed, before drawing definitive conclusions.

Soft tissue mineralization and calcification about knee, heel and ankle are described in WS cases; it may be a non-specific local response or only a sign of a complex underlying disease. In fact, periarticular calcification in the hands and feet, which may be present in scleroderma and systemic lupus erythematosus, also occurs in Werner syndrome, where it represents an important secondary symptom (Leone A et al, 2005).

Controversy still exists concerning which is the degree of brain involvement.

While individuals with Werner syndrome may have central nervous system complications due to arteriosclerosis, they do not appear to be particularly susceptible to Alzheimer disease or other degenerative neurologic alterations different from those arteriosclerosis related (Martin et al 1999).

Cognitive changes are not typically observed.

There is no gender dominance; in WS patients, the male:female ratio is believed to be 1:1. In the International Registry of Werner Syndrome, females are slightly over-represented, probably because they are more likely than men to present for medical care and tend to have more concern about a youthful appearance.

The prevalence of Werner syndrome varies with the level of consanguinity in populations.

In the Japanese population, where this condition could be due to marriage between consanguineous (very often in the past) and to the background high rate of *WRN* mutations, the frequency ranges from about 1:20,000 to 1:40,000, based upon the frequencies of detectable heterozygous mutations (Satoh et al 1999).

This is most likely due to founder mutations in the Japanese population (i.e. mutation 1, mutation 4, mutation 6), as shown in a recent review (Rossi et al. 2010).

The prevalence in the US population is unknown, but may be of the order of 1:200,000 (Martin et al 1999).

Clinical and differential diagnosis

Clinical assessment and diagnostic criteria for WS were originally indicated by Goto (1981) and then re-proposed (Nakura et al. 1994) to establish 3 different degrees (“definite,” “probable,” or “possible”) of diagnosis.

These indications were revised in 1997 by Goto with the proposal of the presence of at least four of the following primary findings:

- Consanguinity
- Characteristic facial appearance and body habitus
- Premature senescence
- Scleroderma-like skin changes
- Endocrine-metabolic disorders

At the moment, for a clinical diagnosis is necessary to show 3 of 4 cardinal signs (onset after age of ten years) (<http://www.ncbi.nlm.nih.gov/sites/GeneTests>; Leistratz 2007):

- Bilateral cataracts

- Characteristic dermatological pathology (tight skin, atrophic skin, pigmentary alterations, ulceration, hyperkeratosis, regional subcutaneous atrophy)
- Characteristic *facies*, described as "bird-like" feature of the face (i.e., the nasal bridge appears pinched and subcutaneous tissue is diminished)
- Short stature
- Premature graying and/or thinning of scalp hair
- Parental consanguinity (third cousin or closer) or affected sibling
- Positive 24-h urinary hyaluronic acid test, when available

These cardinal signs are seen in more than 95% of the molecularly diagnosed cases (Huang et al. 2006); but besides the first characters, patients also show secondary and additional signs:

- Type 2 diabetes mellitus
- Hypogonadism (secondary sexual underdevelopment, diminished fertility, testicular or ovarian atrophy)
- Osteoporosis
- Radiographic evidence of osteosclerosis at distal phalanges of fingers and/or toes
- Soft-tissue calcification
- Evidence of premature atherosclerosis (e.g., history of myocardial infarction)
- Neoplasms: mesenchymal (i.e., sarcomas), rare (e.g., unusual sites of melanomas and osteosarcomas (Goto et al 1996, Ishikawa et al 2000)), or multiple neoplasms
- Abnormal voice or voice change (high-pitched, squeaky, or hoarse)
- Flat feet.

The International Registry of Werner Syndrome uses these findings to establish a "definite," "probable," or "possible" diagnosis pending molecular genetics confirmation.

Definite diagnosis: all of the cardinal signs and two others.

Probable diagnosis: the first three cardinal signs and any two others.

Possible diagnosis: either cataracts or dermatologic alterations and any four others.

Diagnosis is excluded when onset of cardinal signs and further symptoms are manifested before age ten years, except for short stature, which is typically caused by lack of the usual adolescent growth spurt.

A correct differential diagnosis is very important for the patients because of the existence of other diseases, whose signs could appear similar to Werner syndrome, taking difficult the right identification of the pathology. These include other progeroid syndromes, with and without increased cancer susceptibility, like as:

- a) Laminopathies, a group of disorders caused by mutations of the type V nuclear intermediate filaments, lamin A/C, encoded by the *LMNA* gene (Broers et al. 2006). A subset of WS patients do not show mutations at the *WRN* locus, but show heterozygous amino acid substitutions in the heptad repeat region of *LMNA*. For this reason, this condition is called "atypical WS".
- b) Mandibuloacral dysplasia (MAD), a rare disorder characterized by short stature, beaked nose, small recessed chin, short fingers, thin, slanted shoulders and hyperpigmented skin and caused by *LMNA* mutations (Novelli et al. 2002).
- c) Hutchinson–Gilford progeria syndrome (HGPS) is a childhood onset progeria caused by a unique splicing mutation of the *LMNA* gene (Eriksson et al. 2003). HGPS patients develop an aged appearance (characteristic facial features, alopecia, loss of

subcutaneous fat, and short stature) by their second to third year of life. Complications of cardiovascular disease usually lead to death between the first and second decade of life, with the mean age of death being 13 years.

HGPS can be distinguished from WS by the age of onset, characteristic facial features and general appearance.

d) Rothmund–Thomson syndrome (RTS) and Bloom syndrome (BLM) due to other helicase proteins defects.

Molecular diagnosis

In the past and until few years ago, were evaluated urinary and serum concentration of hyaluronic acid, that may be increased in some individuals with Werner syndrome (Tollefsbol & Cohen 1984, Goto 1997, Tanabe & Goto 2001). This could be useful to support the diagnosis, but today this testing is considered cumbersome and nonspecific, and thus not recommended.

At the moment, the molecular diagnosis of WS uses to combine nucleotide sequencing (PCR from genomic DNA and RT-PCR from m-RNA of different tissues), necessary to identify the real mutation, with Western blot analysis (WB) (Huang et al. 2006), useful to demonstrate the presence/absence of a normal/truncated protein.

The increased availability of molecular approach has replaced urinary hyaluronic acid testing for diagnostic purpose; today these two methods are currently used by the International Registry of WS (Tab. 3).

Sequence analysis	Western blot analysis
Mutation detection 90% of the affected individuals	Used to determine the effect of mutation on WRN protein
No <i>WRN</i> predominant mutation in Caucasian WS patients	Detection of truncated protein
No large introns or regulatory regions of <i>WRN</i> are analyzed	Revelation of loss of the protein

Tab. 3: Summary of Molecular Genetic Testing Used in Werner Syndrome

Hereditary

Heterozygotes of a *WRN* mutation are asymptomatic and do not appear to be at increased risk for WS-specific symptoms.

The risk for the offspring of an individual with WS to develop the disease is negligible unless the affected individual and his/her reproductive partner are consanguineous.

In Japan, where the frequency of heterozygotes may be as high as 1/150, the risk of WS in the offspring of an affected patient is still lower than 1/500. Siblings of individuals with WS may be affected and may wish to be tested. Siblings old enough to be clearly asymptomatic may also choose to be tested for carrier status.

Construction and evaluation of a pedigree will allow the identification of family members who may be affected or at risk of being carriers of a *WRN* mutation.

Molecular biology: the gene and protein

On a genetic point of view, WS is caused by the mutations at the *WRN* locus on chromosome 8 (8p12-p11.2).

WRN, the main gene associated with Werner syndrome, spans more than 250 kb and consists of 35 exons, 34 of which are coding exons (Yu et al. 1996, 1997).

Exons ranged in size from 68 bp to 767 bp, the largest being the 3' exon which contains a TAA stop codon. The region most highly homologous to RecQ-type helicases was contained in exons 14–21 (Fig. 8) (Matsumoto et al 1997).

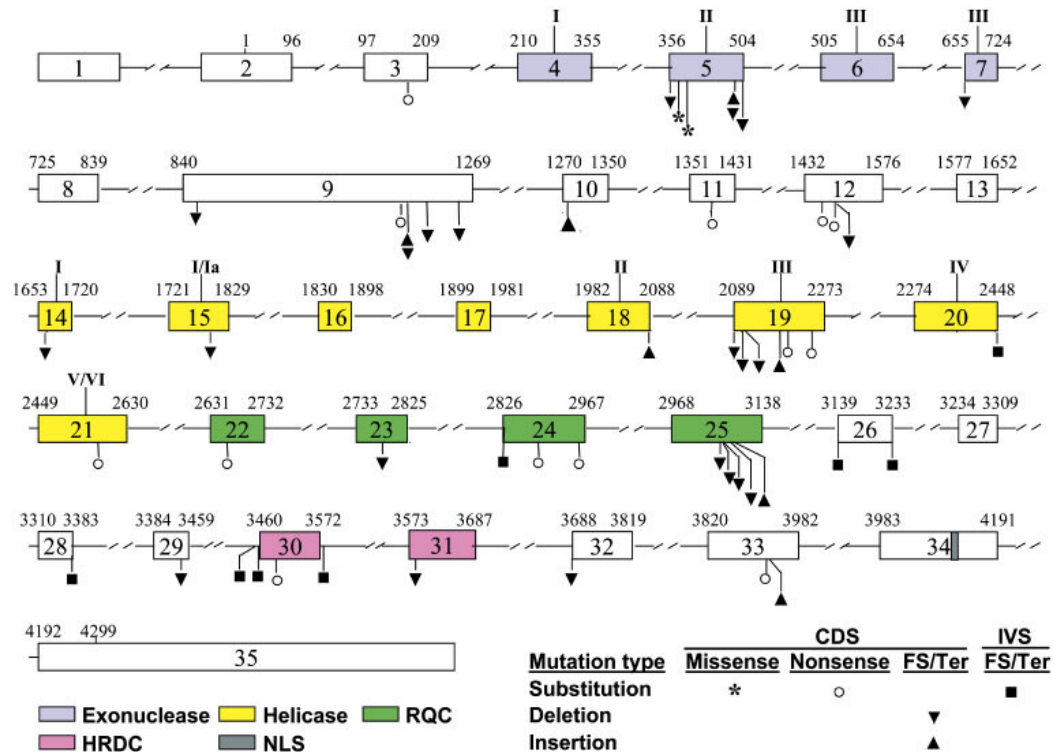


Fig. 8: Schematic representation of *WRN* nucleotide sequence. Exons are designated as boxes 1-35.

Introns are indicated by thin lines. The numbers above the exons indicate the nucleotide positions in cDNA.

The localizations of the functional domains are colored.

CDS, coding sequence; FS/Ter, frameshift and premature translation termination; IVS, intervening sequence.

I-VI indicate the motifs in exonuclease and helicase domains (Huang 2006).

WRN gene encodes one of the RecQ helicase family proteins, WRN, ubiquitously expressed in different tissues, in particular in glandular tissues like as pancreas and gonads.

The normal product has 1,432 amino acids.

The central region of the *WRN* protein contains the consensus domains of RecQ type helicases (Gray et al 1997) and the N-terminal region contains the exonuclease domain (Huang et al 1998) (Fig. 9).

Moreover, there are two consensus domains in the C-terminal region whose functions have not been completely elucidated:

- RecQ C-terminal conserved (RCQ) region
- Helicase RNaseD C-terminal (HDRC) conserved region (Morozov et al 1997).

This C-terminus contains a highly acidic transactivation activity, which functions between the regions containing exonuclease and helicase activities (Ye et al. 1997; Balajee et al. 1999).

The RCQ domain contains two functional regions: a zinc finger motif, which may be involved in the regulation of helicase enzymatic activity by modulating DNA binding as well as protein folding of WRN helicase, and a winged helix motif (WH), region for a unique interaction DNA substrate during the base-separation.

The zinc finger motif and protein-binding domain (DPBD), which covers the WH motif, may play a role in directing the WRN protein to the site of action (Hu et al 2005).

The HDRC region, of unknown functional importance, is speculated to be involved in protein-DNA interactions.

The C-terminus contains also the nuclear localization signal (NLS) that is unique to mammalian RecQ family members; so, for its presence, WRN-GFP fusion proteins can reach nuclear and nucleolar localization in human cells; and furthermore, it may serve as a binding site for the other interacting proteins (Matsumoto et al. 1997) (Fig. 9).

This type of conditional localization is probably important for the protein functions, and could be mediated by post-translational modifications of WRN (Moser et al, 1999).

Early gel filtration chromatography studies indicated that purified full length WRN exists as a trimer (Huang et al. 2000), but on the other hand, current electron microscopy data indicates that WRN is likely found as a dimer in solution. It behaves as a tetramer when is complexed with DNA (Compton et al. 2008), but unwinding DNA, WRN acts as a monomer (Choudhary et al. 2004).

Together these results suggest that WRN's oligomeric state may be dependent on whether or not it is catalytically active and how it is interacting with DNA.

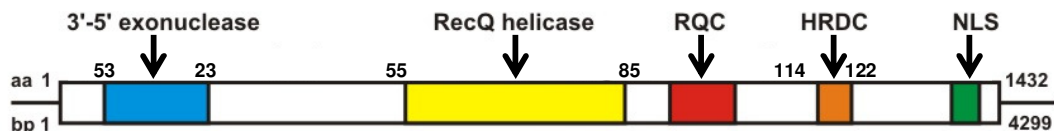


Fig 9: WRN protein and its structural and functional domains.

WRN protein activities and functions

WRN has different ATPase actions: 3'-5' helicase, 3'-5' exonuclease and single-stranded DNA (ssDNA) annealing activities.

Although the biological importance of ssDNA-annealing activity remains to be determined, it has been proposed that *in vivo* this activity might facilitate strand migration during recombination or replication fork movement at the site of DNA damage (Wu L et al. 2006).

All the RecQ helicases proteins have a common helicase domain with seven conserved motifs, which bind and hydrolyze ATP. In particular, WRN helicase activity is structure specific and just requires the energy from ATP hydrolysis to unwind complementary strands of DNA with a 3'-5' polarity (Muftuoglu et al 2008).

WRN helicase specifically unwinds only structures involving DNA metabolic intermediates; these include forked and flap structures (intermediates in DNA replication and repair), bubble structures (intermediates in DNA repair and transcription), Holliday junction structures (intermediates in DNA recombination) and G-quadruplex DNA and D-loop structures (associated with telomere DNA), all of which represent intermediates in DNA replication and repair processes (Opresko et al. 2003).

It unwinds DNA-DNA double strands as well as DNA-RNA double strands.

But, unlike other helicases, WRN also has intrinsic 3'–5' exonuclease activity that preferentially digests single strands in complex DNA structures, such as double-stranded DNA (dsDNA) with mismatched ends or bubbles (Brosh et al 2006); degrading substrates with a 5' overhang, but WRN 3' exonuclease can also degrade blunt-end and various other normal DNA structures in addition to those that contain bubbles and mismatches (Shen and Loeb, 2000), or initiate DNA degradation from a nick or a gap in dsDNA.

WRN exonuclease is active on many of the same substrates that can be unwound by WRN helicase activity, suggesting that the two activities might be coordinated either within a single polypeptide, or by interaction with other WRN molecules or proteins (Kamath-Loeb et al. 1998).

These different activities might be particularly useful during DNA replication, where both nicked and gapped substrates are plentiful; in post-replication repair; and in other repair pathways.

Indeed, WRN protein is capable of a multitude of functions.

Biochemical and cell biological studies, have already suggested that this protein is involved in so many pathways, explaining some aspects of the various physiological changes due to its alterations.

However, the precise molecular mechanisms by which mutations in *WRN* cause the WS phenotype remain unknown.

Recovery from replication fork stalling

The focus of many recent investigations has largely been on the response of WRN deficient cells to the replication disruptions, and the role of this helicase in recovery from replication-dependent DNA damage (Fig. 10). In various experimental studies there have been signs for a role of WRN protein in the prevention of Single Strand Breaks (SSBs) at replication forks and conversion into Double Strand Breaks (DSBs) (Christmann et al. 2008).

In the absence of WRN, stalled replication forks are processed via a compensatory pathway, causing DSBs formation (Franchitto et al. 2008). It is clear that WRN acts to prevent and to protect cells from DSBs formation that can occur as a result of replication fork stalling and collapse.

Moreover, it should be considered that WRN is involved in the response to replication fork stalling induced also by agents that generate crosslinks within the DNA.

When damage is encountered at the fork, replication halts and the fork regresses into a chicken foot structure. In this secondary structure, the lagging strand serves as a template for leading strand synthesis.

Subsequently, WRN can mediate reverse branch migration of the chicken foot to bypass the damage, which can then be repaired by alternate pathways (Fig. 10) (Sharma et al. 2004).

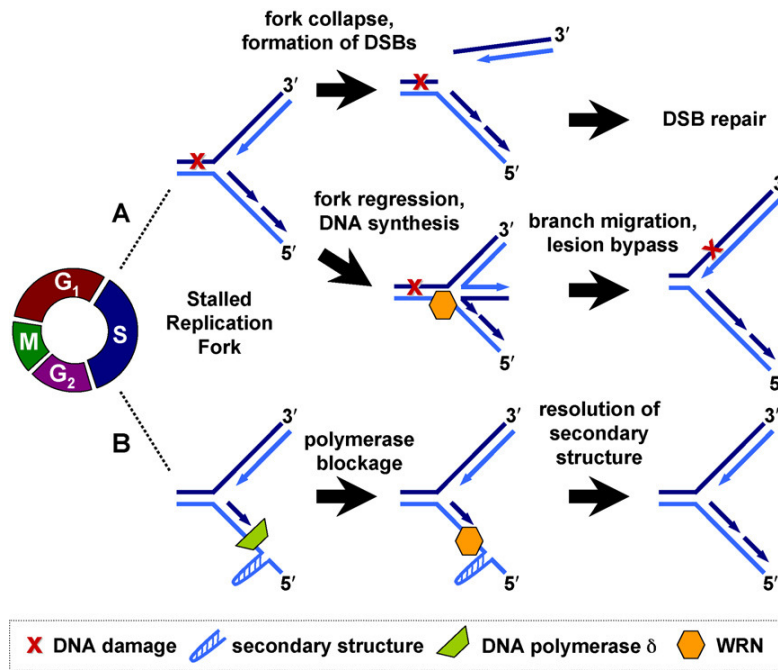


Fig. 10: Roles of WRN in S-phase at stalled replication forks. (A) WRN may participate in repair of double-strand breaks (DSBs) following DNA damage-induced replication fork collapse. Alternatively, WRN may function is to regress the fork and allow for synthesis bypass of DNA damage. (B) Secondary structures which block DNA polymerases may be resolved by WRN (Rossi et al. 2010)

S-phase checkpoints

During S-phase WRN helicase co-localizes with replication foci and interacts with both ATM and ATR, which are mediator-proteins of signaling checkpoint, activated in response to DNA damage.

In response to replication-dependent interstrand crosslink-induced DSBs, WRN facilitates initiation of the intra-S-phase checkpoint through ATM activation.

Many results indicate that WRN plays an important role in reducing DNA damage stress sustained during S-phase.

When cells going to unrolled division, characterized by an accelerated S-phase, which may lead to formation of abnormal replication structures, it is conceivable that WRN may function in repair of these wrong structures. Instead, in WS patients cells, may be because of dysfunction of truncated proteins, it is possible note a certain slowness of the replication cycle and a tendency to remain a longer time in S-phase.

These indications have been demonstrated in cultured fibroblasts WS cells, which have shown a very limited capacity to proliferate.

WRN deficient cells are more susceptible to DNA breaks suggesting that WRN protein is involved in maintenance of fragile sites, in addition to resolve DNA damaged structures and facilitate polymerase replication at fragile sites (Pirzio et al. 2008)(Fig. 10).

Multiple roles of WRN in DNA repair

Double Strand Breaks Repair pathway (DSBR) is induced by external insults (*e.g.*, ionizing radiation) as well as internal causes (*e.g.*, endogenous oxygen radicals) and repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR) processes (Hoeijmakers JH. 2001b).

WRN is known to physically and functionally interact with two key proteins involved in NHEJ: Ku and DNA-PKcs (Yannone et al. 2001; Cooper et al. 2000).

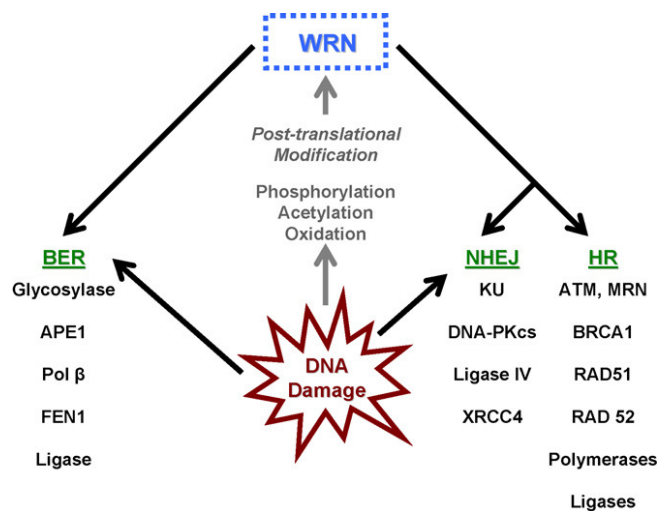


Fig. 11: Roles of WRN during DNA repair. Schematic diagram depicting WRN interactions with various repair proteins in multiple pathways following DNA damage. DNA damage also leads to post-translational modifications of WRN which may regulate its roles in DNA repair. (Rossi et al. 2010)

DNA-PKcs protein enhances the exonuclease activity of WRN and enables it to digest some substrates which WRN alone cannot process.

It is suggested that WRN is probably not an essential component of NHEJ, but is more likely acting as an accessory protein during the DNA repair process and is involved in alternative NHEJ pathways.

Studies both *in vivo* and *in vitro* indicate that the roles of WRN in a variety of DNA processes are mediated by post-translational modifications, as well as several important protein–protein interactions (Bohr 2008; Kusumoto et al. 2007).

In fact, the helicase and exonuclease activities of WRN are inhibited by oxidation, DNA-PK serine/threonine phosphorylation and c-Abl tyrosine phosphorylation.

Interestingly, the WRN helicase and exonuclease activities can work in a coordinated manner on the same substrate and both are often modified by phosphorylation and by binding to numerous interacting proteins (i.e. Ku complex, p53, replication protein A) (Lee et al 2005) like those indicated in Fig 11.

It is presumed that the interactions with all these proteins can determine correlations with at least 3 different DNA repair systems: BER, NHEJ, and HR.

So, WRN protein can potentially unwind or digest aberrant DNA structures accidentally generated during various DNA processes, repair DNA breaks and also regulate DNA recombination by unwinding or digesting intermediate DNA structures.

Taken together, these results suggest that *in vivo* WRN may participate in many aspects of DNA metabolism, including DNA replication, homologous recombination, transcription, telomere maintenance and repair pathways or in a combination of these pathways, such as recombination during replication (Bohr 2005).

These findings are consistent with the notion that WRN plays a role in maintenance of genomic stability (Ozgenc & Loeb 2005, Hisama et al 2006).

In fact, cells or cell lines of WS patients show the presence of cytogenetic and/or molecular genetics instability. Much more, WS cells have selective sensibility to only 2 DNA damaging agents but not to others, and don't show deficits in any of the major DNA repair pathways.

Cellular senescence and telomere maintenance in WS cells

Primary cells from WS patients (like as fibroblasts and lymphocytes) have a limited capacity to proliferate and show replicative senescence in culture at much earlier passages than is characteristic of control cells. This rapid senescence is not generally well correlated with the length of telomeres (the sequences that cap the ends of the linear chromosomes) in the WS cells.

Most of the organisms, telomeres consist of long repetitive G-rich and C-rich DNA strands, the ribonucleoprotein telomerase, and telomere binding and associated proteins.

In mammals, telomeres are composed of double-stranded tandem repeat sequences followed by a single-stranded short 3'-overhang, which invades the double-stranded region to create a loop structure. Loss of telomeric repeats or loss of protection by telomere-associated proteins triggers telomere dysfunction.

Association of proteins that recognize chromosome termini like as broken DNA ends for repairing can form telomere dysfunction-induced foci and can induce cell cycle arrest, senescence, or apoptosis (di Fagagna et al. 2003; 2004).

Aging and premature aging diseases, like Werner Syndrome, due to the lacking function of a helicase protein, are also frequently associated with telomere attrition.

It would appear that telomeres in cells from WS are “unstable” rather than simply being prone to excessive shortening. Some studies suggest that WRN has a crucial role for maintaining the structural integrity of the telomere.

Indeed, WRN protein localizes to telomeres only during S-phase of the cell cycle and can interact with telomere binding proteins, including TRF1 and TRF2 (Telomeric Repeat-Binding Factor 1/2, which are not catalytic proteins, but essential for recruitment of protein essential for telomere maintenance and/or repair), Ku and POT1 (Protection Of Telomeres 1, that strongly stimulates WRN and BLM helicase) (Opresko PL et al, 2002, 2004b, 2005). Thus, WRN might help to resolve aberrant DNA structures that tend to form as the replication fork progresses through telomeric repeats. However, in the absence of WRN, telomeric recombination is elevated (Laud PR, et al, 2005).

Several studies showed that TRF2, regulates the exonuclease activity of WRN; in fact, Machwe and collaborators (2004) showed that TRF2 recruits WRN to process telomeric ends that form into T-loops, a proposed loop-back structure in which the G-rich single-stranded end invades internal telomeric sequences to generate a three-stranded structure (a form of D-loop). Dissociation of D-loop structures is required for release the invading strand and for DNA replication at telomeric regions (Fig. 12).

The function of one of this protein complex, POT1, is to bind the single-stranded G-rich portion of telomeres; it plays a role in capping telomeric ends, preventing them being recognized as DNA damage, and also enhances D-loop unwinding of WRN by maintaining the unwound strands in a melted state.

Moreover, WRN has a role in restart of stalled replication forks, an important mechanism for continuing the replication process following DNA damage. Thus, WRN and POT1 can cooperate with each other to resolve the G4 structures at telomeres (Fig. 12).

Besides biochemical evidence of WRN interaction with other telomere associated proteins that are necessary for maintaining the telomere length, there is cellular evidence of a role for WRN in telomere processing; like as further supported by accelerated telomere loss displayed in WS cells (Crabbe et al. 2007).

Since WRN interacts with both these named proteins, it may be also required to suppress the unusual recombination intermediates at telomeres and be implicated in oxidative DNA damage repair at telomeres (Opresko et al. 2008).

The presence of repetitive sequence of triple guanines makes telomeric DNA prone to oxidative damage, mainly repaired by the BER process, but WRN is believed to take part in BER because it physically interacts with several of this complex system involved in the restoration of correct form of DNA.

This result also indicates a role of WRN in resolving complex structures at damaged telomeres in order to accelerate the activities of the repair enzymes.

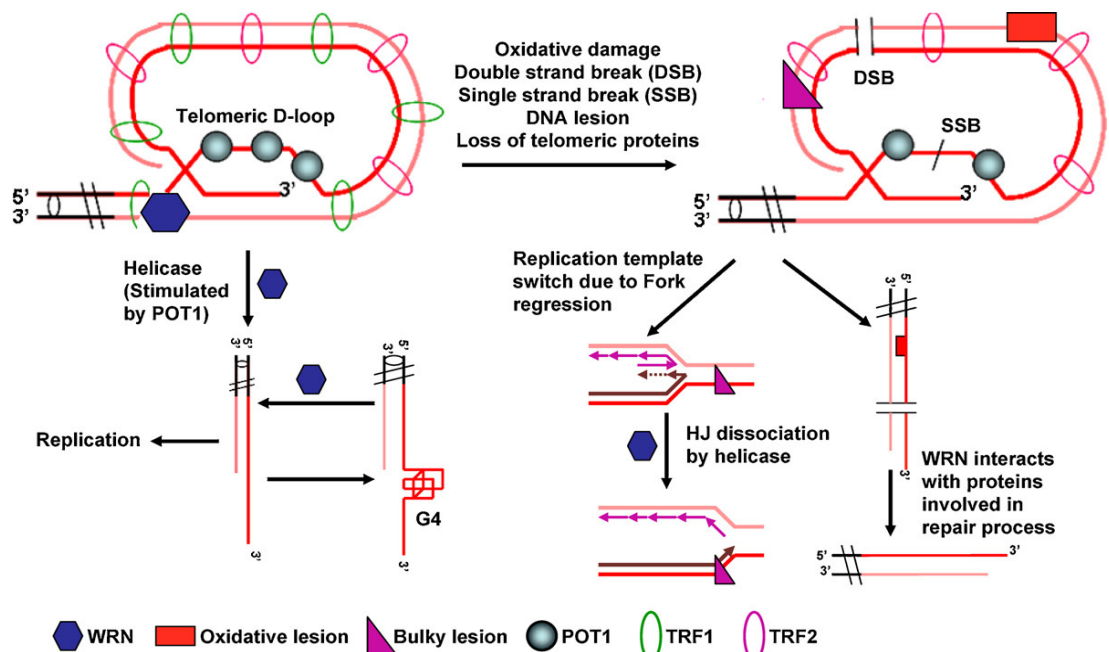


Fig. 12: Role of WRN in replication, recombination, and repair processes at telomeres. (Rossi et al. 2010)

Pathological mutations of *WRN* gene, and consequences on the protein

At the moment, 71 different disease-causing *WRN* mutations have been identified and published by Authors around the world (Goto et al. 1997; Oshima et al. 1996; Uhrhammer et al. 2006; Yu et al. 1997, revision of Friedrich et al. 2010).

They are distributed across the open reading frame of the gene and thorough till few years ago was retained a greater presence of alterations in the C-terminal half, today it seems not be correct. In fact, it has been seen that the alterations can interest this gene in all its regions.

But, at today, 87 *WRN* mutations are registered and available at International Werner syndrome website: <http://www.pathology.washington.edu/research/werner/database>. They include:

(a) 21 nonsense mutations that change an amino acid codon to a stop codon and cause the termination of protein translation;

- (b) 25 small insertions and/or deletions, which lead to reading frameshift and subsequent termination of protein translation;
- (c) 17 substitutions at splice junctions causing the skipping of exons and a consequent frameshift;
- (d) 5 missense mutations that cause the amino acid change in the protein
- (e) 1 intragenic large deletion
- (f) and 2 genomic rearrangements extending beyond the *WRN* locus. (Friedrich et al, 2010). (Probably there is no evidence of pathogenicity for the other 16 *WRN* mutations, and their real effects are unknown).

WS patients have homozygous or compound heterozygous variants; but a not little portion of affected individuals have shown only a single mutant *WRN* allele; it is possible for the incomplete screening of the *WRN* gene open reading frame, the lack of further analysis (i.e. genomic rearrangements) or for the presence of modifying elements yet not discovered.

The majority of these mutations result in completely truncations of the *WRN* protein, subsequently becoming functionally null. The only and simple loss of protein was associated with the syndrome of WS, for some time, this seems to be a satisfactory explanation of why we did not observe obvious differences in phenotype due to different common mutations of *WRN* gene (Huang et al. 2006).

The most frequent Japanese *WRN* mutation is c.3139-1G>C [IVS 25-1G>C] (mutation 4) that causes the skipping of exon 26, it accounts for approximately 67% of the Japanese *WRN* mutations and is thought to be due to a founder effect (Goto et al. 1997; Satoh et al. 1999), because in a Japanese cohort of WS patients this mutation resulted characteristic of a haplotype that was different by the ancestral one, thus uncommon for the Japanese population.

Another frequent mutation is the nonsense mutation in exon 9, c.1105C>T, p.R369X (indicated as mutation 6). This change accounts for approximately 20-25% of the mutations in Caucasian and in Japanese WS cases (Goto et al. 1997; Huang et al. 2006). Also mutation 6 seems to be arisen independently, but more recently, on virtually the same haplotype as mutation 4 (Matsumoto et al, 1997).

The same condition was described for mutation 1 (c.3913C>T, p.R1305X) third more frequent mutation in Japan patients, suggesting that it had a single and independent founder effect too. These data induced to retain that these haplotypes might predispose individuals to mutations in *WRN* gene.

[The simple numbering of the mutations cited here, was based on the first molecular results on the patients (Ishikawa et al 1999) and was maintained for several years; then, after the discovery of many other changes, the previous one has been abandoned over time, although some items still indicate it].

The fact that most other *WRN* mutations, both within and outside the Japanese population, are associated with different haplotypes indicates that the “Japanese haplotype” is neither necessary for the major contributor to new mutations in the *WRN* gene (Goto M et al 1997).

Another founder mutation was reported in Sardinia, where the Werner syndrome prevalence higher than in Japan [1:100,000 in Japan and 1:59,000 in Sardinia (Piras et al. 2004)]. This is a deep intronic mutations (c.2089-3024A>G; p.V697fs), that create a new splice donor site between exon 18 and 19, and have been identified in 18 WS patients from multiple families (Masala et al. 2007).

The large number of WS patients in Sardinia may have at least two explanations. First, the poor connections between Sardinia and the Italian mainland and in addition, even internal communications on the island itself were hampered; second, the common and frequent usage of consanguineous marriage. Recently new characteristic mutations have been indicated like as originated in Morocco, Turkey, The Netherlands and elsewhere (Friedrich et al, 2010). While is not retained specific to a particular ethnic group, the p.R732X mutation in exon 19, reported in France for the first time, was seen mostly in Italian WS patients (patients studied at University of Siena and described separately in this work). p.Q1165X mutation in exon 30 was mainly observed in Turkish patients, and a p.R1305X mutation in exon 33 was predominantly found in Japanese WS patients (Friedrich et al, 2010).

The known mutations found in WS patients are summarized in Fig. 13.

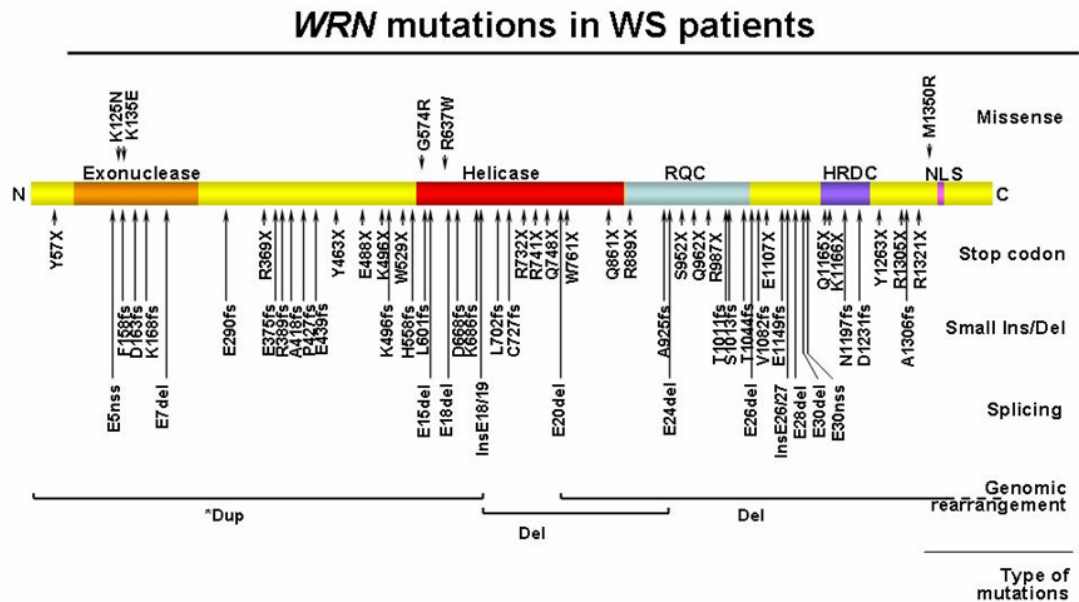


Fig 13: WRN mutations in WS patients. A diagram of the full-length wild-type WRN protein is shown. Known functional domains are marked with *darker shades*; mutations are grouped according to canonical classes. Splicing mutations are indicated by the affected exons. Genomic rearrangements, either deletion (*Del*) or triplication (*Trip*), are shown at corresponding protein locations, with regions extending beyond this figure in *dotted lines*. Splicing mutations that result in identical exon skipping are indicated with little points.

The truncation of WRN due to the nonsense mutations, insertions and/or deletions and splice mutations described above, causes the elimination of the nuclear localization signal (NLS) at the C-terminal end of the protein (Suzuki et al. 2001; Yamabe et al. 1997) which is not able to reach its natural localization.

Many of these truncated mutant proteins may be incorrectly folded as well, thus promoting proteolytic destruction. A second mutation-dependent mechanism may operate at the level of WRN transcript stability (Moser et al. 1999).

In fact, with the loss of the nuclear localization signal, the mutant mRNAs and the resulting mutant proteins exhibit shorter half-lives than do the wild-type mRNA, because they result in new molecules so unstable that they are soon degraded (Yamabe et al 1997).

Although almost all mutations reported so far in WS patients have generated a truncated protein, recently, other missense mutations have been described [Gly574R; M1350R] (Friedric et al 2010) as well as two other missense mutations [K125N; K135E] (double missense mutations in a single subject) have been identified in exonuclease domain (Huang et al. 2006), appearing to affect the stability of the protein rather than enzymatic activities or its localization domain.

5.

6. Mouse models

The phenotype of RecQ-helicase-deficient mammalian cells typically includes genomic instability and sensitivity to DNA-damaging agents, which is consistent with the idea that RecQ helicases are 'guardians of the genome'.

In contrast to cellular features, the phenotypes of some RecQ-deficient mice are difficult to interpret. One example is that animals with homozygous deletion of *BLM* die during embryogenesis (Woo LL et al. 2007).

Heterozygous *BLM* mice display some features of human BS patients including increased frequency of SCE; however, this phenomenon is only observed in humans that are homozygous for a *BLM* mutation. Mice expressing varying levels of BLM were generated; in these animals, the level of BLM correlates inversely with the frequency of SCE, the extent of genomic instability and cancer susceptibility (Woo LL et al. 2007).

Whether a similar correlation exists in humans remains to be determined.

Although WRN helicase has several activities in human cells, Wrm-null mice do not show any phenotypes of human WS and are phenotypically normal.

However, mice in which both WRN and Terc (which encodes telomerase RNA component that in humans interacts with WRN protein) have been mutated, the last generation (mTerc^{-/-}Wrm^{-/-} mouse) showed a complex WS-like phenotype (Du X et al. 2004), characterized by premature death, hair graying, alopecia, osteoporosis, type 2 diabetes, cataracts and increased incidence of non-epithelial malignancies (such as osteosarcomas and soft tissue neoplasms) (Chang et al. 2004).

In particular, this mouse model showed accelerated replicative senescence and accumulation of DNA-damage foci in cultured cells, as well as increased chromosomal instability and cancer, particularly non-epithelial malignancies.

These genetic data indicated that the delayed manifestation of the complex pleiotropy of WRN deficiency is related to telomerase shortening.

It is important to note that these features do not represent deterioration of aging phenotypes observed in the telomerase null mouse, but reiterate the specific phenotypes of WS patients (Hanada and Hickson, 2007).

Together, these indications strongly support the hypothesis that Wrm-null mice do not show human WS phenotypes, as they have long telomeres, and to manifest the premature aging symptoms, critically short telomeres are required.

These data indicate that the latent clinical features of WS might be caused by telomere shortening but also that WRN might play an important part in telomere maintenance, because it is evident that WRN and telomerase work together to maintain the telomere integrity, and WRN is strongly involved in telomere dynamics.

Another characteristic sign of Werner syndrome is the incidence of cataracts, which might be mediated by a direct association between WRN and NBS1 (Cheng et al. 2004), a protein involved in DSBR.

Interesting, mice deficient in NBS1 develop cataracts at an early age and demonstrate aberrant lens-fiber differentiation (Yang et al. 2006).

Additional RecQ-homolog-deficient mouse strains are currently being constructed and characterized, and it is hoped that the phenotypes of these mice will provide novel insights into the biological functions of mammalian RecQ proteins.

7. Atypical Werner syndrome: mutations on *LMNA* gene

In about 20% of cases conforming at least possibly or probably to Werner syndrome according to the International Registry criteria, no mutation in the *WRN* gene is detected, indicating genetic heterogeneity for this disease (<http://www.wernersyndrome.org>; Moser et al. 1999; Chen et al 2003).

Four of 26 such patients (15%) were shown to carry dominant missense mutations of the *LMNA*, gene that encodes the nuclear envelop proteins Lamin A and Lamin C, produced via alternative splicing.

Patients affected with atypical form of WS seem to have early onset (early 20s) of aging phenotypes and show an accelerated rate and a more severe course of progression and associated cardiomyopathy than those who are affected by classic WS (Chen et al. 2003). But, in these patients, absence of bilateral ocular cataracts and diabetes is also common.

While it is clear that *LMNA* mutations are linked with atypical WS, the mechanisms for the biological manifestation of these mutations has yet to be elucidated (Rossi et al. 2010).

LMNA gene is also involved in Dilated Cardiomyopathy and Cardiac Conduction Defects, in Lipodystrophy Disorders and other pathologies.

In particular, its dominant missense mutations are also responsible for other inherited syndromes of aging, notably the more severe Hutchinson- Guilford syndrome (HGS) (Chao H, Hegele RA, 2003), characterized by partial, progressing to total alopecia, loss of subcutaneous fat, stiffness of joints, bone changes, development of severe atherosclerosis and death generally between 6 and 20 years of age as a result of complications of cardiac or cerebrovascular disease.

Besides all what described above, a surprisingly large number of patients referred with potential WS lack either WRN or LMNA mutations.

These patients represent 31% (41 of 130 individuals) of all patients submitted to the International Werner Syndrome Registry, such as indicated in a review, recently published (Friedrich K et al. 2010).

This group likely contains mistaken diagnoses; instances where WS was caused by WRN gene silencing (promoter hyper-methylation); individuals with mutations in proteins required for WRN function; or subject affected by novel progeroid syndromes.

8. Clinical aspects of Werner syndrome on the patients

Characteristic changes on the skin and interpretation

WRN, as is the case for virtually all other helicases, is likely to function as part of a multiprotein complex.

If WRN protein functions as part of such a complex, mutation or inappropriate modification of any component in addition to WRN could confer a common biochemical defect on human cells and promote the appearance of WS.

In WS, many pathological aging features appear most dramatically in the skin.

By the second decade of life, most WS patients show sclerodermatous skin changes such as atrophy, pigmentation, and depigmentation. Hyperkeratosis also occurs, often associated with ulcers on the feet and ankles (Goto M et al. 1981). Ulceration often becomes severe and amputation is required in certain cases.

Histological studies have shown that the appendages and epidermis are atrophic for a much reduced number of cells, and that the thickness of epidermis is reduced in WS patients. It is important to report that WRN can modulate the physiological cellular senescence, normally induced by MYC protein action (Grandori C et al, 2003). It is possible that MYC-dependent differentiation may be highly accelerated in the skin development of WS patients, which results in accelerated aging in this organ. Therefore, skin aging in WS patients may be due to the combined effect of a low proliferation potential and accelerated MYC-dependent differentiation.

However, this remains mere speculation at this stage.

WS patients characteristically develop skin ulcers in the extremities, especially in the regions of the malleoli of the ankles, the Achilles' tendons, and the heels and toes (Faragher et al. 1993) however, the molecular mechanism underlying ulcer formation has not yet been completely elucidated. These ulcers are refractory to any conservative treatment and often require amputation of the limbs.

The occurrence of the cancer in WS patients

Since 1940, Werner syndrome has been associated with an excess of cancer of various and unusual types (Epstein 1966).

The major characteristics for the type and incidence of tumors in WS cases were already reported in 1996 by Goto, who for first described Werner like as a multiple cancer predisposing syndrome. His and following different studies reported that:

- 1) The rate of occurrence of no epithelial tumors was ten time the usual rate
- 2) No epithelial tumors included bone (osteosarcomas) and soft-tissue tumors, malignant melanoma, leukaemia and myelodysplastic syndrome
- 3) Gastrointestinal cancer, lung cancer, and prostate cancer were rare, whereas epithelial neoplasm like as meningioma (Goto et al 1996) and also thyroid cancer were frequent (Ishikawa et al 1999).

The occurrence of multiple tumors in these persons is believed relatively common and in particular for mesenchymal sarcomas, such as soft tissue sarcoma and malignant melanoma, and for epithelial tumors (Futami et al. 2008).

When benign meningioma and preleukemic disorders were excluded, the ratio of epithelial to non-epithelial cancer was 1:1 in WS patients vs the ratio 10:1 in the general population. The following ones are mesenchymal malignant neoplasms found associated with Werner syndrome: fibrosarcoma, fibroxanthoma, leiomyosarcoma, rhabdomyosarcoma, malignant melanoma (rare in Japanese, but not among those with WRN), malignant fibrous histiocytoma, nerve sheath sarcoma, liposarcoma, osteogenic sarcoma and uterine myosarcoma (Epstein et al, 1966; Goto et al, 1981; Sato K et al, 1988; Hrabko et al, 1982). In the report of Goto (1996) the most frequent epithelial cancer in Japanese WS was resulted thyroid carcinoma, accounted for 14% of neoplasms in Japanese WS subjects as compared with 3% in non-Japanese cases.

Another cancer very often associated with this syndrome was osteosarcoma; but while it normally has a peak in late adolescence, in WS patients occurred at a mean age of 45 years. The high frequency of soft-tissue sarcoma and osteosarcoma in a just connective-tissue disorder raised the question of a casual relationship. No excess of these sarcomas was been reported in other connective-tissue disorders, and the excess cancer in WS was not limited to these sarcomas (Goto et al. 1996).

There were no substantial differences in age distribution according to tumor site.

It is also noteworthy that carcinomas that often affect the elderly people, such as those of the lung, colon, or prostate, seldom occurred in WS patients.

Basal cell epithelioma is a common cutaneous tumor of normal aged individuals, so it should be present in progeroid syndromes. However, at today, reports of these cases in Werner syndrome are very rare.

Besides a relative high incidence of malignant melanoma, only one case of basal cell epithelioma was reported in literature (Morita K and Miwa M, 1995), although other 4 skin cancer cases had been described before.

Generally the prognosis for cutaneous malignant melanoma with WS may be poorer than that without WS.

According to the literature, whereas the frequency of malignant tumors varies from 5,6% to 25% the meningioma results the most frequent benign neoplasm in Werner syndrome, both in Japanese and Caucasian populations. In particular, meningiomas in Werner syndrome have a higher frequency in males and occur at younger age than those of the general population (10-30 years before).

Pathological and biochemical studies suggested that abnormal metabolism in connective tissue should have been the most likely explanatory theory of abnormal mucopolysaccharides and fibroblasts in Werner patients (Chang et al, 2004).

The fact that there was a great proliferation of collagen fibers in meningioma could be determined by the aberration of connective tissue metabolism that was implied in pathogenesis of Werner Syndrome.

In other studies, the occurrence of hematopoietic malignancies in patients with WS, however, was extremely rare; there were reported only few cases with these conditions (Undar L et al, 1994), myelodysplastic cancer, associated with WS. In fact, only 4 cases (3 men and 1 woman) with acute leukaemia were reported in the English literature (Undar L et al. 1994; Goto M et al 1981; Björnberg A 1976; Tao et al. 1971) and two additional cases in Japan.

Only 6 cases of lung adenocarcinoma were reported in literature (3 male and 3 woman, median age 52 years) demonstrating that these pathologies are extremely rare in association with WS (Yamanaka A et al, 1997, Ohnishi S et al. 2010). Well differentiated adenocarcinoma was present in 2/4 patients.

Association studies: polymorphisms linked to cancer predisposition

To our knowledge, the specific correlation between somatic mutation in the *WRN* gene and the subsequently specific cancer type has not yet been identified.

Several epidemiologic studies have been done searching the single nucleotide polymorphisms (SNPs) of *WRN*.

For example, two most common polymorphisms, Leu1074Phe and Cys1367Arg, both located in the C-terminus of WRN protein, where there is the binding site for BRCA1, have been comprehensively investigated and found out associated with the risk of some diseases including aging, osteoporosis, and most important, familial breast cancer. Moreover, the concomitant presence in a woman of both *WRN* C1367R and *P53* P72R polymorphisms seems to increase the breast cancer risk in comparison with the presence of these polymorphisms alone (Wirtenberger et al. 2006, Wang Z et al. 2009); this due to the creation of an interference with the apoptotic function of p53, that might be the cause of the breast cancer phenotype or contribution to cancer susceptibility in general.

This polymorphism (C1367R) was previously described as a protective factor of some diseases associated with WS, such as myocardial infarction and type 2 diabetes mellitus (Ye et al. 1997; Hirai et al. 2005) and recently, has been shown to be a protective in soft tissue sarcomas, and malignant fibrous histiocytoma (MFH) (Nakayama et al. 2008).

However, no association was found in a recent work between the presence of 3 *WRN* polymorphisms - V114I (rs2230009), L1074F (rs2725362), C1367R (rs1346044) – and predisposition of colorectal cancer (Frank et al. 2009).

Roles and effects of epigenetic alterations in Werner syndrome

Recent studies indicate that expression of WRN and other premature-aging-associated proteins might be regulated epigenetically (Agrelo 2006, Opresko 2007).

In various types of primary tumor-cells, in many tumors of mesenchymal and epithelial origin, including those commonly observed in WS patients, the *WRN* gene is inactivated by CpG island promoter hyper-methylation (Agrelo et al. 2006).

Thus, this process is common and correlates with the silencing of *WRN* gene determining lower levels of WRN protein expression. These not genetic alterations have been shown in colon cancer, breast cancer, non-small cell lung, gastric tumors, and leukaemia, demonstrating that epigenetic inactivation of the *WRN* gene has important clinical relevance.

In addition, when cancer cells are treated with DNA demethylating agents to reverse hypermethylation of the *WRN* promoter, the defect in WRN exonuclease and the cancer phenotype are rescued.

Other genes involved in premature aging; for example, lamin A/C (*LMNA*) gene expression has been observed by several Authors as modulated by molecular post-transcriptional modifications.

Epigenetic regulation, specifically hypermethylation of tumor suppressor genes, is a newly and tightly recognized mechanism for modulating genes involved in aging and cancer (Esteller et al. 2007); clearly, this is an area of considerable interest for further study.

In primary tumors, hypermethylation of the *WRN* promoter is common and correlates with lower levels of WRN protein expression. This change inactivates the gene and results in increased chromosomal instability, another characteristic of WS patients cancer cells.

The knockdown of WRN protein expression induces cell death and growth arrest in several human cancer cell lines, consistent with a tumor-suppressor like role of *WRN* gene. Moreover, inactivation of WRN in cancer cells increases susceptibility to the cytotoxic effects of topoisomerase inhibitors commonly used as chemotherapeutic agents (Futami et al. 2007).

This is an important aspect that should be considered for understanding other events leading to tumorigenesis, but also for a better use of this knowledge to therapeutic aims.

9. Roles of WRN protein in DNA repair, senescence and cell transformation

Genomic damages that normally are suffered by human cells from environmental, mutagen and toxic agents, could be so important in WS affected individuals, because WRN deficiency leads to hyper-oxidation and rapid and premature accumulation of other negative metabolites, which normally accumulate primarily in older individuals.

Oxidative DNA base modifications are caused by endogenous reactive oxygen species (ROS), normal by-products of mitochondrial oxidative phosphorylation and other metabolic processes, and are removed by Base Excision Repair system (BER) (Fig 14).

The involvement of WRN in BER is evident from the fact that WS cells are sensitive to hydrogen peroxide (von Kobbe et al. 2004), and WRN siRNA treated human primary fibroblasts also accumulate increased damage after oxidative stress (Szekely et al. 2005).

As an example, increased protein oxidation might explain high frequency and early onset of cataracts in WS patients, possibly due to oxidation of lens proteins.

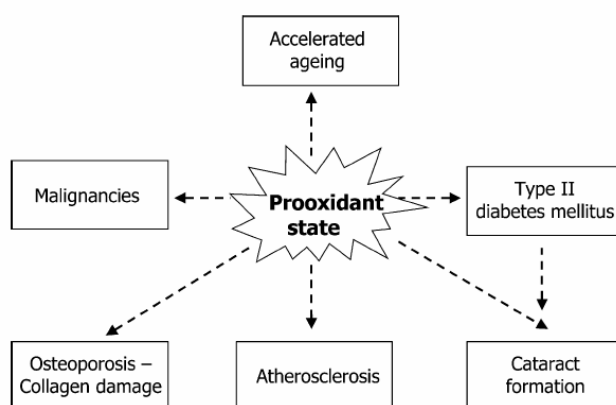


Figure 14: Oxidative stress may be regarded as a central event related to the occurrence of the clinical abnormalities observed in WS (Pagano 2005).

High levels of oxidative stress and oxidative DNA damage have been seen associated with increased risk for sarcomas; thus, hyper-oxidation and dysfunctional or decreased BER activity could potentially explain the high incidence of this type of cancer in WS patients (Kansara et al. 2007).

Moreover, WRN interacts with the tumor suppressor p53 (Brosh et al. 2001; Sommers et al. 2005; Yang et al. 2002), so as to activate repair process on damaged DNA and/or initiate apoptosis mechanism; in fact, when there is present a WRN dysfunction, subsequently WS cells have attenuated p53-mediated apoptotic response (Spillare et al. 1999), leading to a not controlled replication and to cellular transformation.

It is presumed that in the absence of this helicase protein, stalled forks either could be resolved through a complicated process of recombination and deletion events, or would cause the cell cycle arrest in the S-phase, resulting in the slower replicative and cell division capacity.

It has been suggested that improper maintenance of telomere length, and consequent telomere dysfunction (and cellular dysfunction) are strictly linked to chromosome instability, accelerated aging and accelerated manifestation of malignant tumors in general (Chang S et al. 2004).

As confirmation of this hypothesis, human fibroblasts with loss of WRN were described with extremely limited capacity to proliferate, in particular, with an *in vitro* life span of less than 20 population doublings (Marton E et al. 2006). And moreover, as a consequence of inability to reinitiate stalled replication forks, the cells became also sensitive to certain DNA-damaging agents and showed an elevated frequency of deletion mutations *in vitro*.

It is presumable that cells lacking or with a truncated protein (and therefore not functional) could be hypersensitive to exposure of environmental genotoxic factors, showing increased cell cycle arrest and cell death compared to wild-type cells.

One example of these damaging elements could be Chromium, an environmental genotoxin known to affect DNA replication through formation of interstrand crosslinks, which can inhibit polymerase elongation of the DNA, and also through creation of DSBs during S-phase, and can lead to replication fork collapse (O'Brien et al. 2002; Reynolds et al. 2009). Other physical DNA and cellular toxic agent may be irradiation.

Survivors of atomic-bomb exposure have chromosome instability and aberration that last for decades. Medical X-ray exposures increase the risk of certain types of cancer that overlap those in excess in WS patients: at low doses, AML and thyroid carcinoma; at high doses, soft tissue sarcoma, osteosarcoma, and meningioma. High risk of cancer after medical radiation exposure of WS patients, as well as also other environmental carcinogen environmental factors is a possibility considered plausible, but difficult to test, because of the lack of information (Goto 1996).

How much this phenomenon can be determinant is very hard to demonstrate; the fact that there was no excess of WS individuals with cancer near Hiroshima or Nagasaki can induce to think that not only the molecular and cellular damages due to the environment, but their co-existence with *WRN* gene mutations or other genetic alterations, are responsible for the development of cancer in these latter cases.

Many findings have demonstrated that with the loss of WRN helicase there are genetic instability and mutagenesis, that determine a decrease of the cellular proliferative capacity, due to "low fidelity" DNA repair system and metabolism, creating "intermediate phenotypes" that may limit the number or the quality of cells needed to complete or maintain tissues or structures.

These data confirm the relevant roles and functions of WRN protein, which could represent an important link between defective DNA repair processes, cell senescence, apoptosis and onset and development of cancer (Opresko PL et al, 2003).

10. Previous attempts of genotype - phenotype correlation

The chronologic order of the onset of various signs and symptoms is similar in all individuals with Werner syndrome, regardless of the specific *WRN* mutations (Epstein et al 1966, Tollefsbol & Cohen 1984, Goto 1997).

But the specific type of tumor occurring in different WS subjects might depend on the particular type of *WRN* mutation that they present.

This theory and new findings clearly contradict the original assumption that all identified *WRN* mutations result in truncation of the nuclear localization signal of WRN protein and thereby act as null mutations.

The first and may be the only one attempt to create a correlation between genotype and cancer phenotype was made by Ishikawa and collaborators in 1999, considering only the onset and development of thyroid carcinomas in WS Japanese patients.

In that report were obtained genetic data of only 23/150 patients with thyroid carcinoma. They found that the female-to-male ratio for the patients with cancer was 2.3:1, vs. 6.1:1 in the general Japanese population.

More, the follicular to papillary thyroid carcinomas ratio was 3:1 for WS patients vs 6:1 in healthy people (follicular cell type was predominantly in the patients, 48%; while papillary type was the most frequent in the general Japanese population, 78%).

Furthermore, they tried to formulate a link between mutation's site and the type of cancer developed by the patients, since that in individuals of Japanese descent, papillary carcinoma had been associated with an N-terminal mutation, whereas follicular carcinoma had been more frequently observed with a C-terminal mutation.

This could suggest a possible genotype-phenotype relation in terms of thyroid carcinoma histology, although it could have occurred by change due to the small number of case (Fig 15).

The mutations found patients with thyroid carcinoma were typical of those found overall among Japanese patients with WS, and thus it appeared that no particular mutations have a link to thyroid tumorigenesis, especially.

They also marked the fact that there was, however, a difference in “*tumor spectrum*” between Japanese and whites.

In fact, no increase in the frequency of thyroid carcinoma had been reported among whites; although they have increased frequencies of soft tissue sarcoma, osteosarcoma, and benign meningioma, as do the Japanese.

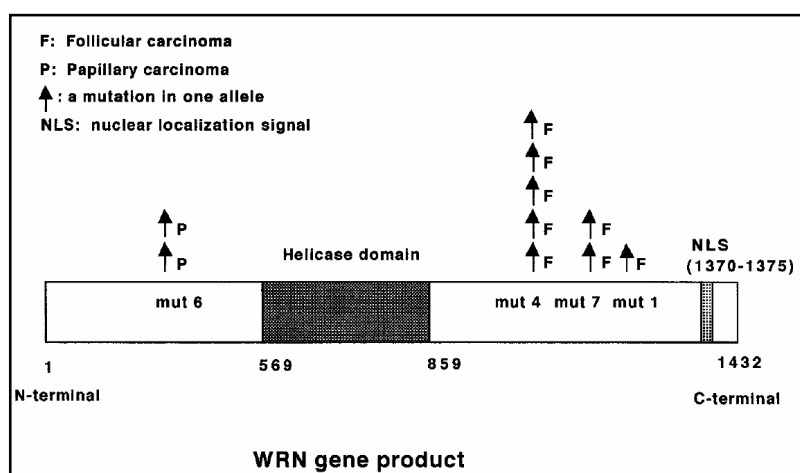


Fig 15: Structure of the *WRN* gene product and positions of mutations found in Japanese Werner syndrome cases with thyroid carcinoma (Ishikawa et al 1999). The shaded area is homologous to RecQ helicase.

According to the Authors, possibly the excess of thyroid tumors in Japanese people were due to environmental disparities, particularly a diet rich or deficient in iodine and may be to the higher presence of mutation 4 (c.3139-1G>C; p.G1047fsX1061), possibly playing a key role in the genesis of thyroid carcinoma in WS patients.

In fact, that continues to be the most common in Japanese, but not in whites, with the same syndrome.

This was an attempt of correlation, but a thorough clinical evaluation has failed thus far to reveal any difference in symptoms among patients with different mutations (Matsumoto, Imamura, 1997).

This finding clearly contradicted the original assumption that all identified *WRN* mutations result in truncation of the nuclear localization signal of *WRN* protein and thereby acted as null mutations, so leading to the same protein dysfunction.

In fact, it was possible to indicate that *WRN* mutations were likely to be functionally equivalent null alleles. Therefore, it was “unlikely” that a different spectrum of *WRN* mutations alone could explain the elevated risk of thyroid carcinoma in Japanese patients.

The Authors believed that until the functions of wild and mutant *WRN* proteins will be understood fully, it was natural to keep open the possibility that different mutations may cause different phenotypes.

These indications were important and decisive for new attempts of genotype-phenotype correlations.

In Ishikawa report in 2000, the distribution of osteosarcomas (OS) by skeletal site was unusual in all ten patients with Werner syndrome respect those reported for the general population: much more in lower limbs, especially present in foot/ankle bones (Fig. 16 and Fig. 17).

This could have some implications for the pathogenesis of the tumor, because it was previously demonstrated that “atypical” OS (arising in short and flat bones) showed subtypes other than the three major histological types, more often than “typical” OS (arising in long bones) (Machinami R 1979).

The marked difference of anatomic sites between WS and the comparison group raised questions about the pathogenesis of OS in WS.

Table II. Histological Subtypes of Osteosarcoma in Werner Syndrome and the Comparison Group (Hospital Series)

	Werner syndrome	Comparison group
No. of cases	10	36
Age range (years)	35–57 (mean 48.0)	35–57 (mean 45.3)
Gender (F:M)	5:5	18:18
Histological subtype		
Osteoblastic	5 (50%)	14 (39%)
Chondroblastic	2 (20%)	4 (11%)
Fibroblastic	2 (20%)	4 (11%)
Other ^{a)}	1 (10%)	14 (39%)

^{a)} Including well-differentiated, telangiectatic, small-cell, MFH (malignant fibrous histiocytoma)-like subtypes and so forth.

Fig 16: Histological subtypes of osteosarcoma found in Werner syndrome patients, in comparison with hospital series (Ishikawa et al. 2000).

The legs were affected at older ages by ulcers, gangrene, and wasting of soft tissue. The ankles were very thin. They thought that genetic disorder caused anatomic changes that concentrate weight-bearing at the ankle, which perhaps led to osteosarcoma in a predisposed host. The vulnerability in Werner syndrome was indicated by the reports of OS at other unusual sites in Japanese patients (two radius, one patella) (Fig. 17).

The concurrence of clusters of two types of cancer at the ankle/foot suggested a pathogenesis in common.

It might be that among genetic disorders with high risk of OS, the excess at the ankle/foot occurred only in WS because of its late-stage wasting of the legs (Ishikawa Y, et al 2000).

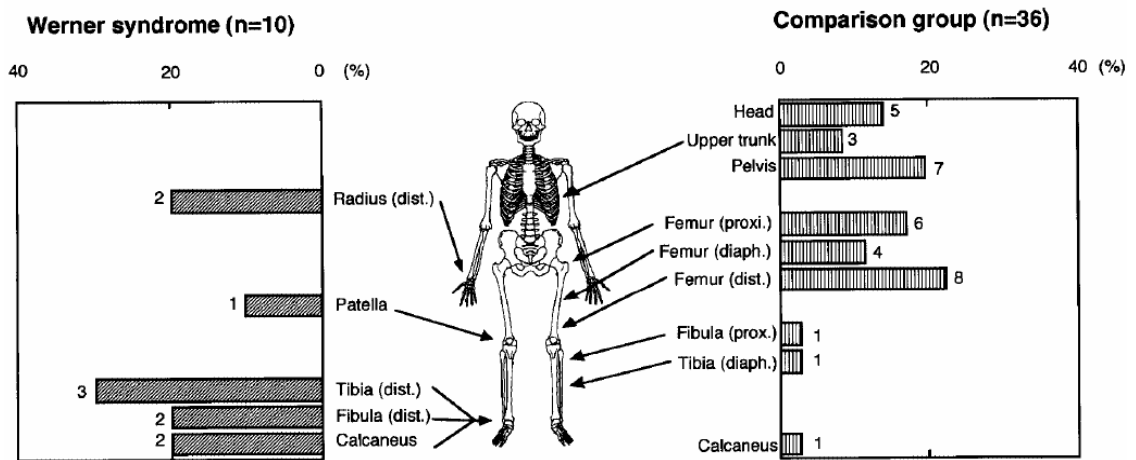


Fig. 17: Comparisons of sub-sites of osteosarcomas arising in Werner syndrome and in the comparison group in Ishikawa report (2000).

11. Present study

This study was performed through 3 steps:

- 1) Review of the literature reports concerning all the available information on Werner syndrome and other progeroid diseases; to make inference concerning the incidence and the occurrence of different neoplasms in particular in WS subjects.
- 2) Report of two additional cases of Werner syndrome, who were admitted to the Dept of Neurological and Behavioural Sciences of the University of Siena.
In particular, in these two patients the germ-line mutation was detected, mainly for diagnostic purposes and for evaluation of the risk of developing the same condition in other members of the family.
- 3) Extended survey and prevalence analysis of subjects with WS in a Geriatric Hospital in Milan, Italy, that is the larger geriatric institute in Europe, to test how many individuals with Werner syndrome or related syndromes are present among elderly people. At the same time it was possible to compare the incidence of degenerative diseases of the central nervous system (CNS), such as Alzheimer's (AD), among all patients in this hospital, and in particular the relative rare incidence of different types of neoplasms in these degenerative disorders.

The literature review

WS cases with cancers and with a precise mutation detection

More than 70 mutations of *WRN* gene have been reported in the literature, most of which are named only as allelic pathologic variants, useful for the genetic and molecular diagnosis, indicating them as cause of the onset and development of characteristic clinical symptoms of the disease. However, little is known about their involvement in the evolution of the disease, such genetic alterations involving a classical clinical aspect and which further lead to a more severe phenotype, to also cancer onset and development.

Since the first reports of the disease (Epstein et al 1966), it was evident that many subjects with WS had associated neoplasms, usually occurring at an earlier age in comparison to the general population.

However, in most of these reports, only clinical associations between subjects with phenotype characteristics of WS and cancer were reported, whereas a precise description of the type and site of the germ-line mutation responsible for the syndrome was lacking. In fact, the RecQ helicase gene was discovered and cloned in 1996, and accurate genetic tests were developed later.

Therefore, the cases reported before 1996-2000 interval are not useful to detect specific genotype-phenotype correlation, even if they can be of interest to assess the broad range of associated neoplasms.

Instead, some recent papers could report some Werner cases associated with different types of cancer and with a definite molecular (genetic) diagnosis.

Our literature review is concerning all the available information on Werner syndrome, including etiology, clinic, pathology, molecular biology, genetics, epidemiology, etc.

All these data were obtained from papers and website databases, and should be assessed carefully, in particular to make inference concerning the incidence and the occurrence of different neoplasms in WS subjects.

The major information are reassumed in the table under (Tab. 4), following by a little description of further clinical and pathological characteristics of the patients, cited in relative references. In some cases, there are added other clinical information correlated to the diseases, described here following.

patient	age	sex	facies	WS dermatology	cataracts	stature	aged hair	family	diabetes	osteoporosis	secondary symptoms	diagnosis	nucleotide change *	protein notation *	predicted protein	cancer case reference
1	33	F	yes	yes	yes, 27y	short	yes	yes	yes		vulvar cancer	definite	c.1165delA	p.R359fsX332	331 aa	Vidal V 1997, Uhrhammer NA 2006
2	48	M	yes	yes	yes	1,67 m	yes	yes		yes	cancer (summary table)	definite	c.3244_3245delG / c.1309C>T	p.V1082fsX1091/p.R637W		Uhrhammer NA 2006
3	45	M	yes	yes	yes, 31y	1,55 m	yes, 32y	no			soft tissue calcification, cancer (summary table)	definite	c.3789C>G	p.Y1263X	truncated protein	Uhrhammer NA 2006
4	35	F									osteoblastic sarcoma at patella, FTC, uterus carcinoma	definite	c.3139-1G>C	p.G1047fsX1061	1060 aa	Ozawa M 1990, Ishikawa Y 2000
5	37	M	yes	yes	yes, bilateral	1,70 m	no	yes	yes		fibroblastic type of osteosarcoma at the radius	definite	c.3139-1G>C	p.G1047fsX1061	1060 aa	Murata K 1999, Ishikawa Y 2000
6	50	M						yes			osteoblastic type of sarcoma at tibia, melanoma	definite	c.1105C>T	p.R369X	368 aa	Nishimoto H 1990, Ishikawa Y 2000
7	55	F	yes	yes	yes, bilateral	1,39 m	yes	yes	yes	yes	soft tissue calcification, osteoblastic type of osteosarcoma at calcaneus	definite	c.1105C>T	p.R369X	368 aa	Isuji Y 2000, Yamaga Y 1995, Ishikawa Y 1999
8	39	M									FTC, MFH	definite	c.3913G>T / c.3139-1G>C	p.R1305X / p.G1047fsX1061	truncated protein	Goto M 1961, Ishikawa Y 1999
9	40	F									FTC	definite	c.3139-1G>C	p.G1047fsX1061	1060 aa	Ishikawa Y 1999
10	42	F									FTC	definite	c.3446delA	p.E1149fsX1161	1160 aa	Ishikawa Y 1999
11	44	F									FTC	definite	c.3139-1G>C	p.G1047fsX1061	1060 aa	Yashiro T 1985, Ishikawa Y 1999
12	43	F									PTC, meningioma	definite	c.1105C>T	p.R369X	368 aa	Sugimoto N 1991, Ishikawa Y 1999
13	56	F	yes	yes	yes, bilateral	1,46 m	poliosis	no			thyroid, benign meningioma	definite	c.3139-1G>C	p.G1047fsX1061	1060 aa	Nakamura Y 2005
14	58	M		yes, ulcers	yes, bilateral	1,49 m	yes	no	no	yes	thyroid adenoma, 3 primary sarcomas (popliteal fossa, MH at thigh, osteosarcoma of calcaneus)		c.3233-1G>C	p.G1047fsX1061	1060 aa	Moser 2000, Nakamura Y 2003, Huang 2006
15	61	F	yes	yes	yes, bilateral	1,40 m	yes	?	yes	yes	papillary pancreas carcinoma	definite	/ c.3913C>T	/ p.R1305X	1304 aa	Shimaoka Y 2007, Matsumoto T 1997
16	50	F	yes								(adreno) cortical carcinoma of the liver, papillary tumor of ureter	definite	c.1105C>T	p.R369X	368 aa	Takazawa R 2004
17	44	F			yes	1,57 m	yes	yes			atrophy of ovaries, 3 primary malignant melanomas	probable	none	no expression	absent (blotting)	Shibuya H 2005
18	?	?	?	?	?	?	?	?	?	?	unique type of osteosarcoma, unclassified	definite	c.3915dupA	p.R1306fsX1318	1317 aa	Goto M 1966, 1997
19	52	M	yes	yes, ulcers	yes, bilateral		yes, 15y	yes	yes		osteomyelitis, lung carcinoma	definite	c.3139-1G>C	p.G1047fsX1061	1060 aa	Ohnishi et al 2010
20	?	?	?	?	?	?	?	?	?	?	thyroid carcinoma unclassified (summary table)		c.3915dupA	p.R1306fsX1318	1317 aa	Ishikawa Y 1999
21	51	F		yes	yes, bilateral	1,45 m	yes				hard tumor near right elbow, MIFH	definite	c.3139-1G>C	p.G1047fsX1061	1060 aa	Sogabe Y et al. 2004
22	52	M		scleroderma	yes		alopecia	yes	yes		diabetes, no evident cancer		c.2194C>T	p.R732X	731 aa	Malandrini A 2000, De Stefano N 2003
23	42	F		scleroderma			alopecia	yes	yes		diabetes, no evident cancer		c.2194C>T	p.R732X	731 aa	Malandrini A 2000, De Stefano N 2003

FTC = Follicular Thyroid carcinoma
PTC = Papillary Thyroid carcinoma
MFH = Malignant Fibrous histiocytoma
MH = Malignant histiocytoma

case negative for genetic analysis of mutation 1-4-6
cases studied at University of Siena
cases found in review literature, with incomplete clinical and personal indications

* revised WRN mutation nomenclature, following the guide-lines of the Human Genome Variation Society and recently published by Friedrich et al. (2010)

Case 1) Vulvar cancer is one of negative conditions traditionally observed in elderly patients and therefore likely to be related to Werner's syndrome. But this is the first WS case reported with this type of cancer, despite the fact that it is a typical cancer of elderly women. Besides the pathological mutation reported in the table, this woman had also another alteration: she was heterozygous for a missense polymorphism or mutation (c.1392G>A, p.Met387Ile); may be the complex genotypic condition was determinant for the particular severe and unusual age-related cancer (Vidal V 1998).

This is probably the third example of urologic and reproductive system cancer reported in the literature (only one case of prostate and one case of bladder carcinoma are found) (Hrabko PR, 1982; Saeki H, 1987)

Case 2) Patient heterozygous for two new mutations. In particular, p.Arg637Trp missense mutation seems likely to disrupt the function of the helicase domain and is the first example of a null missense allele in an affected subject.

The substitution of bulky, uncharged tryptophan may render the protein non-functional by disrupting its secondary structure or interaction with its substrate (Uhrhammer et al, 2006).

Case 3) Part of a collection of data by Uhrhammer and collaborators (2006) among French WS patients (other clinical information not available).

Case 4)-5)-6)-7) Examples of WS patients with osteosarcoma and genetic diagnosis showed in the Ishikawa's review (2000). Not in all the cases it was possible to derive the clinical and pathological information.

Case 5) Hard tumor with erosion and ulcer was found on the dorsal aspect of left forearm of an affected man. It was composed of four histological patterns: a malignant fibrous histiocytoma-like, a desmoids-like, a dermatofibrosarcoma protuberans-like, and a chondrosarcoma-like. The ulna was pressed by the cancer mass.

And it was diagnosed like as a fibroblastic type of osteosarcoma.

This case could be considered a sort of typical example of WS patient with osteosarcoma.

Contrary to general population, where most common sites of osteosarcomas are the distal femoral and proximal tibial metaphyses, and the most common cell type is osteoblastic, in Werner patients is noted the development of this type of cancer in unusual anatomic districts and often they are not constituted by osteoblastic cells (Murata K 1999).

Case 7) The first reported genetic demonstration of Werner case with osteosarcoma.

Mutation 6 (c.1105C>T, p.R369X), leading to the creation of a stop codon, is predicted to produce a truncated protein that lacks enzymatic activity, culminating in the loss of helicase function (Matsumoto et al, 1997). This mutation is confirmed to cause the completely loss of the function of the WRN helicase.

In particular, for this patient there was an atypical position of the osteosarcoma (the calcaneus) and a delay of the occurrence of the neoplasm (55y in the patient vs. 40y in other WS individuals).

It is so interesting case because in WS affected subjects generally there is described an accelerated manifestation of the natural aging process.

However, this lesion demonstrated the typical characteristics of osteosarcoma arising in patients with Werner syndrome; as reported previously (Ishikawa et al. 1996), the average

age of patients who have osteosarcoma in association with WS is 40y and the lesion affects sites that are atypical for osteosarcoma.

Case 8)-9)-10)-11)-12) Summary of data derived from the report of Ishikawa et collaborators (1999), in which they tried to correlate the onset and cell type thyroid carcinoma with the type and nature of particular *WRN* gene's mutation. Description of their results has been discussed previously.

Case 13) In the general population, meningioma predominantly affects women with a female-to-male ratio of approximately 3:2, however in WS patients this ratio is reversed in 8:19. The meningioma described in this woman (like as all the ones reported in WS patients) was intracranial, as well as commonly in the general population (Goto 1996). The incidence of meningioma in the healthy population increases with age (Satoh M et al, 1999); therefore its presence in progeroid syndromes is quite rife, making it the most frequent benign type of tumor found in the patients.

In this case the meningioma was diagnosed at age of 56y, although the average age of patients with meningioma in association with WS is 43y.

This woman, showed also calcification of both Achilles' tendons.

Moreover, this patient had altogether two tumors: a thyroid tumor and a brain tumor.

Until 2008 there were described in the literature at least other 37 WS cases associated with meningiomas (4 of these associated with also thyroid carcinoma) (Tsurubuchi et al. 2008)

Case 14) Is described only one case of Werner syndrome characterized by the occurrence of thyroid carcinoma and subsequently concomitant presence of three kinds of primary sarcomas, osteoarthritic changes in the joints and osteoporosis.

The patient has resulted homozygous for a no common *WRN* gene mutation (also called mutation 20; c.3233+1G>C, p.G1047fs1060). The skipping of exon 26 was found in one French pedigree, due to this substitution from G to C at the splicing consensus sequence in intron 26 adjacent to exon 26 (described by Moser et al. 2000a, and Huang et al. 2006).

This alteration seems to give the same effects on the protein predicted product than mutation 4, the most frequent among Werner syndrome patients.

The first cancers diagnosed were thyroid adenoma (43y), and an osteosarcoma of the right popliteal fossa (43y). Then another tumor, a malignant fibrous histiocytoma of the left thigh was found (50y). This man showed also osteosclerosis of the calcaneous and calcification at the Achilles' tendon. An incisional biopsy indicated a well-differentiated intramedullary osteosarcoma (55y).

Interestingly, almost all the clinical symptoms of this patient were noted before the age of 40 years.

In the literature are described cases with multiple primary tumors in association with Werner syndrome, but only one case with more than 2 non-epithelial malignant tumors (Goto et al. 1996).

However, only three reports of patients with more than three tumors have been published, and none with three primary sarcomas.

In this case, like as others reported previously, there is found a sort of combination of thyroid cancer and various sarcomas, suggesting some special form of carcinogenesis (or carcinogenic risk) in WS (Nakamura Y et al. 2003).

Case 15) Until 2007, this is the second reported Werner syndrome individual with pancreatic carcinoma. The reason for the limited number of these cases in the past seems to be that the mean life expectancy of patients with this syndrome is only 54 years.

Pancreatic carcinoma is generally believed to occur most frequently in men 70 years of age or older and naturally, many patients with this pathology die before reaching the usual age of onset of pancreatic carcinoma.

It was found only mutation 1 (c.3913C>T, p.R1305X) by molecular diagnosis. Considering the recessive inheritance, this affected woman was necessary compound heterozygous for *WRN* alterations, in the same gene (somewhere not investigated) or in other interacting and modulating elements.

She had hoarseness and suffered of cerebral ischemic attack at age 29 years.

She showed also calcification of the Achilles tendon and she was diabetes insulin resistant.

Case 16) This other woman had reported two cancer: a mass in the right adrenal gland, which compressed the liver and the vena cava; and a right ureteral tumor. The patient did not have predisposing factors of urothelial carcinoma that is a type of cancer linked mainly to ambient factors (including cigarette smoking).

On the contrary, adrenocortical carcinoma is a type of cancer linked mainly to genetic disorders, but not to environmental exposure.

Generally urothelial carcinoma occurs in WS; but adrenocortical carcinoma has not been reported before this case (Takazawa R 2004).

Case 17) Though Werner clinical diagnosis could seem clear for this woman, because of the presence of typical symptoms and the concomitant occurrence of 3 primary malignant melanomas, the cause of this condition may be due to an unusual mutation of *WRN* gene in Japan population.

One excisional biopsy specimen of a pigmented lesion on the large pudental lip and other two lesions, identified as pigmented maculae, were found near the toe and the heel respectively; all these skin lesions were diagnosed like as malignant melanomas.

In particular, these signs indicated the proliferation of atypical melanocytes arranged as solitary units at dermo-epidermal junction, and an inflammatory infiltrate was present.

In fact, the patient was negative for 3 of the major mutations in this country (mutation 1-4-6); but, from the blotting assay it had not been possible to detect the protein.

Perhaps other genetic alterations were responsible for the loss of *WRN* protein, such as the absence of expression due to hypermethylation of *WRN* promoter or extremely rapid and complete degradation of the dysfunctional messenger, determining the phenotypic characteristics in this woman (Shibuya et al, 2005).

Case 18) and case 20) The personal and clinical information of these patients were not available, since they belong to a larger collection of cases. Genetic data refer to quotations found in other papers.

Case 19) A man with lung cancer, adenocarcinoma (ADC) (4 cm), designed clinically stage (cT2N0M0), and tumor invasion of pleura, but not lymph nodes. Interesting, histopathological examination revealed mixed cellular patterns; that is, both papillary and solid. These findings correspond to those of adenocarcinoma mixed subtype, with lymphocytic and plasma cell infiltration (Ohnishi et al 2010).

Lung cancer has been very rarely observed in subjects with WS, probably because these patients usually have a shorter lifespan.

Lung cancer develops most frequently in men who are 50 years-of-age or more (Jemal et al. 2003). This represents the sixth case of lung cancer associated with Werner syndrome (4/6 were ADC), and all the patients were over 50 years of age. In previous reports it was not undertaken *WRN* mutational analysis, so the molecular cause of this disease and complications remains unknown.

Seems that there are no differences in the pathological type of primary lung cancer between patients with and without WS, as half of all cases of primary lung cancer in non-WS patients are adenocarcinoma (Harada et al. 2005).

However, there are still very few reported cases of primary lung cancer in patients with WS. Therefore, more experience of primary lung cancer in WS patients is needed before definite conclusions can be drawn.

Case 21) First early aging manifestations in this woman begin to be visible at 20y. In her late forties, she noticed near the right elbow joint a small nodule that had grown over the years. There was a hard tumor, diagnosed as a storiform-pleomorphic type of malignant fibrous histiocytoma (MFH).

The patient showed also subcutaneous calcifications on the bilateral Achilles tendons.

No brain abnormalities (report by Sogabe Y et al. 2004).

This woman carried a homozygous mutation at one base up-stream of exon 26 of *WRN*, mutation 4, (c.3139-1G>C; p.G1047fsX1061), causing a frameshift leading to premature termination.

Case 22) and case 23) Werner syndrome patients come to Department of Neurological Science of University of Siena and admitted care for neurological disease.

Their description is following in the next sections.

Tab. 5: Description of the WRN gene mutations and the number and types of tumors found associated.

Nucleotide mutation	change on the protein	cases	Domain location predicted protein	cancer type associated	cancers reported
c.1105C>T	p.R369X	4 hom	no functional domain, between exonuclease and helicase, stop codon, truncated protein	osteosarcoma	2
				melanoma	1
				meningioma	1
				papillary thyroid carcinoma	1
				papillary tumor of ureter	1
c.1165delA	p.R389fsX392	1 hom	no functional domain, between exonuclease and helicase, stop codon, truncated protein	vulvar cancer	1
c.1909C>T	p.R637W	1 het	missense mutation in helicase domain, not more functional	not definite	1
c.3139-1G>C	p.G1047fsX1061	7 hom 1 het	3' end of RQC domain, stop codon upstream HRCD, truncated protein, without NLS domain	follicular thyroid carcinoma	5
				osteoblastic sarcoma	1
				uterus	1
				malignant fibrous hystiocytoma	2
				meningioma	1
				lung carcinoma	1
c.3233+1G>C	p.G1047fsX1061	1 hom	3' end of RQC domain, stop codon upstream HRCD, truncated protein, without NLS domain	thyroid adenoma	1
				sarcoma	1
				osteosarcoma	1
				histiocytoma	1
c.3244_3245delGT	p.V1082fsX1091	1 het	downstream RQC domain, stop codon upstream HRCD, truncated protein, without NLS domain	not definite	1
c.3446delA	p.E1149fsX1161	1 hom	no functional domain, upstream HRDC domain, truncated protein, without NLS domain	follicular thyroid carcinoma	1
c.3789C>G	p.Y1263X	1 hom	no functional domain, stop codon upstream NLS, truncated protein, without NLS domain	not definite	1
c.3913C>T	p.R1305X	2 het	no functional domain, stop codon upstream NLS, truncated protein, without NLS domain	pancreatic cancer (papillary carcinoma)	1
				malignant fibrous hystiocytoma	1
c.3915dupA	p.R1306fsX1318	2 hom	no functional domain, upstream NLS, truncated protein, without NLS domain	osteosarcoma	1
				thyroid carcinoma	1

Total patients 21

Total mutations associated with cancer 10

Data interpretation:

We found 10 previously described different *WRN* gene mutations, belonging to 21 Werner syndrome patients, which had developed at least one type of cancer. For each mutation are reported the site of change on the WRN protein, the allelic conditions found in the patients (3 subjects in heterozygosis), the number and the type of the cancers found associated (Tab. 5).

Three of the ten mutations that were associated with cancer, were localized in the N terminal region of the WRN protein, whereas the other 7 mutations were in the C terminal region (Fig. 18).

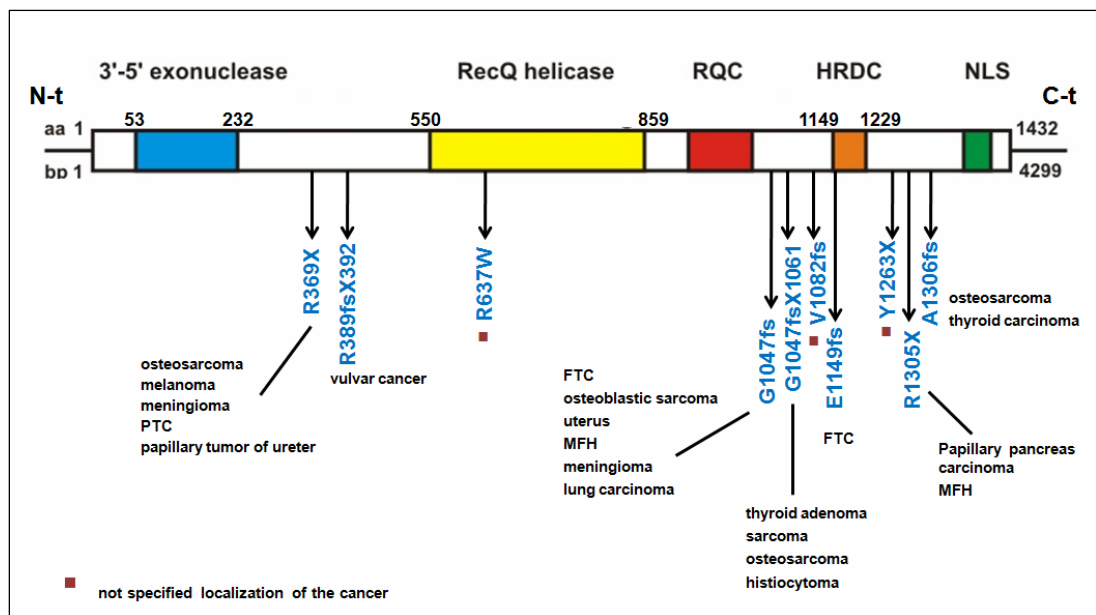


Fig. 18: Localization of 10 *WRN* gene mutations found associated with different types of cancers. The types of the tumors are described above the respective protein changes
 PTC: Papillary Thyroid Carcinoma
 FTC: Follicular Thyroid Carcinoma
 MFH: Malignant fibrous Hystiocytoma.

The major part of these mutations (8/10) hits no catalytic domains, while only two variations were found inside functional regions (RecQ helicase and HRDC domain respectively).

The first domain (RecQ helicase) has the most important enzymatic function for the WRN protein. In fact, it is able to unwind double strands of DNA with a 3'-5' polarity (Muftuoglu et al 2008), such as complex nucleotides structures as forked and flap structures, Holliday junction structures and G-quadruplex DNA and D-loop structures (Opresko et al. 2003).

The HDRC region, of unknown functional importance, is supposed to be involved in protein-DNA interactions.

Otherwise, even one simple aminoacidic change in these catalytic domains is responsible for the stability and correct folding of the molecule, guarantying all enzymatic activities of

WRN protein and consequently its important role and actions during replication and maintenance of the genomic stability.

Furthermore, only one was a missense mutation, whereas all the others were 3 nonsense and 6 frameshift mutations, determining the same type of alteration on the protein product. In fact, nonsense mutations, that change an amino acid codon to a stop codon as well as small insertions/deletions and splice junctions mutations, which lead to reading frameshift or the skipping of exons and a subsequent frameshift, cause the termination of protein translation, resulting in a shorter and truncated molecule.

The truncation of WRN causes the loss of the Nuclear Localization Signal domain (NLS) at the C-terminal end of the protein (Suzuki et al. 2001; Yamabe et al. 1997) and for this region, it is not able to reach its natural localization in the nucleus.

Like as described in the literature, the most part of *WRN* gene mutations has as effect a truncated and null functional protein product.

The most frequent germ-line mutation in our literature review was c.3139-1G>C [IVS 25-1G>C] (mutation 4) that causes the skipping of exon 26. It was the first most common mutation, accounting for approximately 67% of the WRN mutations in Japanese patients (Goto et al. 1997; Satoh et al. 1999).

In our literature review it is present in 15/42 in the total of alleles (35,7%).

The great difference between these data can be explained by the small number of WS cases with genetic and definite diagnosis that we have been able to examine in our research, because of the small number of available reports in the literature (most of which are written in Japanese languages, unfortunately). Another not indifferent reason could be that the frequency values are often derived by association studies or epidemiological studies, whose aims are only to determine the probable group of individuals at risk of development of this disease in that population.

This mutation was particularly frequent in patients living in isolate places, such as Japan, where marriages between consanguineous were usual and was not so present in others, therefore, it is thought to be due to a founder effect.

The second most frequent mutation in Japanese patients as well as in our literature review was the nonsense mutation at amino acid 369 in exon 9, c.1105C>T, often indicated mutation number 6. This change accounts for approximately 20-25% of the mutations in Caucasian and in Japanese WS cases (Goto et al. 1997; Huang et al. 2006).

In our series it is present in 8/42 alleles (19%). Also mutation 6 seems to be arisen independently, but more recently, on virtually the same haplotype as mutation 4 (Matsumoto et al, 1997).

In our literature review, among the 23 patients with cancer associated with the molecular detection of the germ-line mutation there were 9 cases of thyroid carcinoma and 9 cases of osteosarcoma.

In 21 WS patients with a clear description of cancer, thyroid carcinoma totally represents 42% of the cases (9/21 patients).

The most frequent *WRN* mutation found in this group was c.3139-1G>C (mutation 4), followed by the other 4 mutations (1 case for each ones).

The most representative was follicular type of thyroid carcinoma (FTC) (5 cases of 9 patients), very rare remained the papillary type of thyroid carcinoma (much more frequent in the general population), with only one case described in our review (Tab. 6 and Tab. 7). There is a probable association between mutation 4 and the occurrence of FTC in Werner syndrome patients, like as was proposed by Ishikawa and collaborators in 1999 and already described in previous sections.

Association with thyroid carcinoma (23 WS cases with cancer)				
mutation	cases	gender	type of cancer	II diseases
c.1105C>T	1 hom	F	1 PAPILLARY	1 meningioma
c.3139-1G>C	4 hom	F	4 FOLLICULAR	1 sarcoma, 1 uterus cancer
	1 het	M		1 MFH
	1 hom	F	1 UNCLASSIFIED	1 meningioma
c.3233+1G>C	1 hom	M	1 UNCLASSIFIED	3 sarcomas
c.3913C>T	1 het	M	1 FOLLICULAR	1 MTF
c.3915dupA	1 hom	?	1 UNCLASSIFIED	
tot PATIENTS 9	9/23*	39,0%	5 F	4 sarcomas
	9/21*	42,8%	3M	1 MFH
follicular cases	5			2 meningiomas
papillary cases	1			1 uterus cancer
unclassified	3			

* 23 cases with cancer (2 cases with no specified type of tumor)

* 21 cases with a description of the type of cancer

Tab. 6: Correlations between *WRN* mutation and thyroid carcinomas found in our review.

				Histological subtype		
	Male	Female	Mean age	Follicular	Papillary	Unknown
WS thyroid carcinomas in literature (26)	10	16	40 years	11	10	5
WS thyroid carcinomas in our series (9)	3	5	44 years	5	1	3

Tab. 7: Summary table of main characteristics of WS patients with thyroid carcinomas found in our and in Ishikawa (1999) reviews

A similar attempt has been done for osteosarcoma cancer identified in WS patients.

9/21 WS patients with recognized tumors were osteosarcomas (or sarcomatous type of neoplasms), 42,8% of the cases.

In particular, 3 cases of osteoblastic sarcoma were found in association with c.3233+1G>C mutation, one case with c.3139-1G>C (mutation 4) and one case with c.1105C>T (mutation 6). Mutation 4 was also associated with 1 case of fibroblastic osteosarcoma and 1 case of malignant fibrous hystiocitoma (Tab. 8, Tab. 9).

The only most important and evident correlation can be between the presence of *WRN* mutation and the anatomic site of occurrence of osteosarcomas.

In fact, the primary sites of osteosarcomas in WS patients were mainly the lower extremities, whereas osteosarcomas were more common in the upper extremities in the general population (Ishikawa et al. 2000).

In our literature review we found (number and site of osteosarcomas):

1 patella, 1 terminal distal region of radius, 1 terminal region of tibia, 2 calcaneous, 1 popliteal fossa, 1 MH at thigh, 1 MFH hard tumor near right elbow.

Almost all these cancers were associated with calcification of soft tissues, in particular, with Achilles tendon calcification.

Association with osteosarcoma (23 WS cases with cancer)				
mutation	cases	gender	Number and type of cancer	II diseases
c.1105C>T	1 hom	F	1 OSTEOLASTIC	1 melanoma
	1 hom	M	1 OSTEOLASTIC	
c.3139-1G>C	1 hom	F	1 OSTEOLASTIC	1 FTC, 1 uterus cancer
	1 hom	M	1 FIBROBLASTIC	
	1 hom	F	1 MFH	1 FTC
	1 het	M	1 MFH	
c.3233+1G>C	1 hom	M	3 OSTEOLASTIC	thyroid aenoma
c.3913C>T	1 het	M	1 MFH	
c.3915dupA	1 hom	?	UNCLASSIFIED	
tot PATIENTS 9		9/23*	39,0%	3 F
		9/21*	42,8%	5 M
osteoblastic cases	6			
fibroblastic cases	1			
MFH cases	2			
Unclassified	1			
* 23 cases with cancer (2 cases with no specified type of tumor)				
* 21 cases with a description of the type of cancer				

Tab. 8: Association between *WRN* mutation and osteosarcomas found in our review.

				Histological subtype				
	Male	Female	Mean age	Osteoblastic	Chondroblastic	Fibroblastic	MFH	Unknown
WS osteosarcomas in literature (13)	8	5	47 years	5	2	3	2	1
WS osteosarcomas in our series (10)	6	3	36 years	6	0	1	2	1

Tab. 9: Summary table of principal characteristics of WS patients with osteosarcomas found in our review in comparison with previous studies (Goto et al. 1996; Ishikawa et al. 2000).

Meningiomas are the second most common primary neoplasm of the central nervous system, usually benign in nature; however, they can be malignant.

Meningiomas in Werner syndrome have a higher frequency in males and occur at a younger age than those of the general population (Laso FJ et al. 1989).

Pathological and biochemical studies suggest that abnormal metabolism in connective tissue should be the most likely explanatory theory of abnormal mucopolysaccharides and fibroblasts in Werner patients.

The aberration of connective tissue metabolism implied in pathogenesis of Werner syndrome, is closely related to proliferation of collagen fibres in meningioma.

Grade I tumors show either uniform monosomy or a diploid karyotype. Progression of meningioma is correlated with increasing hypodiploidy, showing characteristic chromosomal alteration such as those seen in tissues of WS patients (Marton et al. 2006).

37 cases of central nervous system (CNS) tumors associated with Werner syndrome have been reported until 2008 (Tsurubuchi et al. 2008), including meningiomas in 33 cases.

The meningiomas in Werner syndrome are almost always a benign form and tend to occur in WS subjects about 2 times more frequently in men than in women, and about 10 to 30 years earlier than the same conditions in the general population (Mahmood A et al. 1993).

Twenty-three of the 33 meningiomas were reported in Japan.

The male to female ratio in the patients with meningioma (23:10) may be higher than that in patients without WS (1:2). In the literature, patients with Werner syndrome, were generally younger (mean age, 43y) compared to those without WS (50-60y) (Tab. 10).

We were able to characterize only two patients (two women) with meningioma and a precise detection of germ-line mutation of *WRN* (mutation 4 and mutation 6) (Tab. 11).

WS meningiomas in the literature		WS meningiomas in this study	
Male	23		0
Female	10		2
	tot 33		tot 2
Mean age	43 years		49 years

Tab. 10: Cases of meningiomas associated with Werner syndrome, the values are relative to clinical or genetic examinations.

Association with meningioma (23 WS cases with cancer)			
mutation	cases	gender	II diseases
c.1105C>T	1 hom	F	PTC
c.3139-1G>C	1 hom	F	unclassified thyroid carcinoma
total patients	2/23 *	8,70%	FEMALE
	2/21 *	9,50%	THYROID CARCINOMA
* 23 cases with cancer (2 cases with no specified type of tumor) * 21 cases with a description of the type of cancer			

Tab. 11: *WRN* gene mutations in two affected women with meningioma.

30 cases were single meningiomas, 3 cases multiple meningiomas, and the main sites were the convexity, falx cerebri, and paragittal areas, as in patients without WS. The major histological types were fibroblastic meningiomas, transitional, and meningothelial meningiomas.

Other types of CNS tumor have also been reported, such brain non-epithelial tumors, originating from the ectoderm, like as intracranial astrocytomas (two cases) (Laso et al. 1989), one case of glioma, and one case of spinal neurinoma (Tokunaga et al 1976).

Until 2005, there have been reported 27 WS cases with malignant melanomas. Clinical values described in the report of Shibuya and collaborators are reported in Tab. 12 in comparison with the data relative to the 4 cases of melanoma (3 tumors in an only one subject) that have been found in our series.

Only one of two patients is known the mutation of *WRN* gene, mutation 6 (c.1105C>T).

	Cases	Male	Female	Mean age
WS melanomas in literature	27	18	9	45 years
WS melanomas in our series	2	1	1	47 years

Tab. 12: Data relative to WS patients with melanomas.

We report also the prevalence of mesenchimal and epithelial types of tumors found among WS patients (Tab. 13).

Substantially, the ratio has slightly declined ($12:14 = 0,85$, in previous studies was 1:1), even if this values could do retain a major presence of epithelial forms of malignancies, contrary to was thought in the past for the role and dysfunction of WRN protein in tissues of connectival origin.

Since tumors occurring WS have mostly been detected during the II-III decades of life, they are obviously different (for site and type) from sporadic tumors occurring in the general population.

In other words, tumors associated with Werner syndrome are mostly mesenchimal tumors and melanoma, whereas in the general population most of tumors originate from epithelia and, in particular, from glandular epithelia.

Since most of these epithelial tumors occur after age of 50 years, because the median age of death is in patients with Werner syndrome in 54 years, there is an obvious sampling bias, which is strictly related to the age of the sample.

In fact, a simple explanation could be that subjects with WS don't develop typical sporadic epithelial cancers, because they don't reach the usual age at which these cancers typically occur.

In fact, in the reported series there was a very low incidence of the most frequent cancers such as colorectal cancer, liver cancer (no case reported up now), breast or lung cancer, gastric cancer or HCC (Hepato-Cellular Carcinoma).

Association with mesenchimal cancers		cases	Association with epithelial cancers		cases
c.1105C>T	p.R369X	3	c.1105C>T	p.R369X	2
c.1165delA	p.R389fsX392		c.1165delA	p.R389fsX392	1
c.1909C>T	p.R637W		c.1909C>T	p.R637W	
c.3139-1G>C	p.G1047fsX1061	4	c.3139-1G>C	p.G1047fsX1061	7
c.3233+1G>C	p.G1047fsX1061	3	c.3233+1G>C	p.G1047fsX1061	1
c.3244_3245delGT	p.V1082fsX1091		c.3244_3245delGT	p.V1082fsX1091	
c.3446delA	p.E1149fsX1161		c.3446delA	p.E1149fsX1161	1
c.3789C>G	p.Y1263X		c.3789C>G	p.Y1263X	
c.3913C>T	p.R1305X	1	c.3913C>T	p.R1305X	1
c.3915dupA	p.R1306fsX1318	1	c.3915dupA	p.R1306fsX1318	1
tot 12			tot 14		

Tab. 13: Correlation between WRN mutation and different origin cancers that have been found in our literature review.

The 23 patients with Werner syndrome and with cancer that have been collected and described in this study had shown other typical and clinical characteristics of the primary disease, besides the high incidence of different malignancies.

We report a summary table of them in comparison with the median values found in a recent review on WS patients admitted to the International Registry of Werner syndrome (Huang et al. 2006) (Tab. 14).

Sign or symptoms	Percent frequency	
	Int. Werner Registry (2006)	our series
Bilateral cataract	100% (87/87)	100% (11/11)
Skin alteration	98,6% (72/73)	90,9% (10/11)
Premature graying / hair loss	96,3% (79/82)	90,9% (10/11)
Pinched facial features	96,1% (49/51)	81,8% (9/11)
Short stature	94,7% (71/75)	72,7% (8/11)
Osteoporosis	90,6% 48/53)	45,4% (5/11)
Type 2 diabetes mellitus	70,8% 46/65)	45,4% (5/11)

Tab. 14: Secondary symptoms shown by WS patients.
(The number of the cases where information was available is given in the parentheses)

Results from two patient diagnosed and followed in Siena



This work is also focused on the evaluation of Werner syndrome cases diagnosed and followed up in Siena. We report two WS patients admitted to the Department of Neurological and Behavioral Sciences, University of Siena: a man and his younger sister, 42 and 32 years old at the moment of the diagnosis (Malandrini A et al. 2000; De Stefano N et al. 2003), today respectively of 52 and 42 years of age. Index case was the brother who had shown very early neurological and movement problems (Fig. 19).

Fig. 19
Pedigree of the two affected cases. Their parents are obliged carriers.

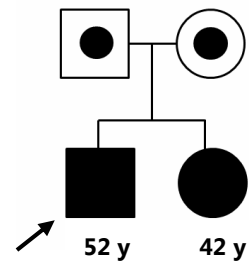


Fig. 20. The patient at 30 years showing sclerodermic changes of the face and distal parts of the limbs, senescent aspect of the face with baldness and beaked nose. Note limb atrophy more accentuated distally.

At 20 years of age he had difficulty in walking caused by pain in plantar region began; at 30 years he underwent surgery for cataract on right eye. General examination showed sclerodermic changes of the face, short stature, and a premature aged aspect, limb atrophy more accentuated distally, calcification of the Achilles tendon, beaked nose and baldness.

A clinical diagnosis of Werner syndrome was done (Fig. 20).

Neurological examination showed moderate dysphonia, slightly spastic gait with moderate hypertonus and normal deep tendon reflex. Psychological test was normal.

Motorevoked potentials showed increased conduction time between the motor cortex and cervical spinal cord.

Brain analysis (MRI plus echo color Doppler) was normal.

At 34-35 years he developed type 2 diabetes mellitus, treated with insulin, which is another secondary symptom of the primary disease.

After few years the clinical and neurological condition began worse, although brain and spinal cord MRI analysis continued to be normal.

His soft tissue calcification increased and diffused to the arteries.

At 40 years his electrophysiological examination showed a worsening of the sensorimotor neuropathy, with respect to the previous examination, with a reduction of myelinated fibers, a marked increase of the endoneurial collagen and neuronal cells with nucleus with unusual shape (Malandrini A et al. 2000).

3 years after, his sister showed diabetes and both had scleroderma, plantar ulcers, dysphagia, alopecia, muscle atrophy and dysphonia.

Their clinical Werner syndrome diagnosis was subsequently confirmed by the identification of a homozygous mutation of the *WRN* gene c.2425 C>T (p.R732X) that had previously been found in a WS French family (Fig. 21). However, in French case, there was no CNS involvement.

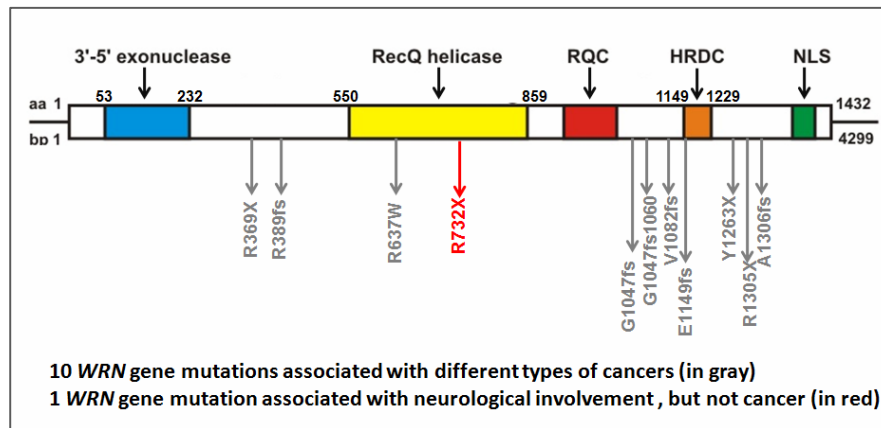


Fig. 21: Representation of the site of p.Arg732X mutation

This alteration causes the formation of a stop codon in the centre of the protein, inside the RecQ helicase domain, the conserved region present in all RecQ helicase protein, which binds and hydrolyzes ATP to unwind only complementary strands of DNA with a 3'–5' polarity (Muftuoglu et al 2008), such as forked and flap structures, Holliday junction structures and G-quadruplex DNA and D-loop structures (Opresko et al. 2003). This region is extremely important for the biologic functions of WRN protein in DNA replication and repair processes.

The c.2425C>T mutation results in a truncated (and so very short) molecule and most importantly, null functional protein. Therefore, that is evident in extensive deletions at non-homologous joining ends, because of WRN incapacity to out-compete with other exonucleases and helicases proteins that participate in double-strand break repair and also stabilize the broken DNA ends (Oshima J et al. 2002).

In this familiar example, CNS involvement was clear in the older brother, who showed mild spastic paraparesis of the lower limbs, weak deep tendon reflexes and bilateral Babinski sign; it was much less evident in the younger sister who showed no neurological symptoms, but only brisk left tendon reflexes on examination.

Cognitive functions were within normal limits in both patients (De Stefano et al 2003).

As previously indicated, tissue reductions have been interpreted as the expression of subtle microscopic or molecular alterations; the low MTR-WM values found in these WS patients is likely retained to reflect a diffuse underlying pathology occurring in WS brain, suggesting that the tissue damage can be present in WS brain in very early disease stages.

This is a very interesting condition: finding decreased neuro-sensorial values in a Werner syndrome 30y patient, without CNS pathologies (asymptomatic), could be indicative for following neurologic problems that not necessarily are present in elderly age or in other WS patients.

And it is in line with the clinical features found in her brother, the major brain tissue reduction and damages are visible only in him, who is older, thus presumably the effects of neuropathology linked to the syndrome could result only when the disease is evident. In his examination were found also decreased levels of NAA that should be interpreted, as in other neurological disorders, as an index of neuro-axonal damage and/or loss.

It must be considered that CNS damage in oldest people tend to be related to a simple senile dementia and interpreted more as a part of an aging than as a real pathological process. And in particular for WS patients, brain atrophy is often showed evident only in the latest disease stage, and for this reason it might be under estimated.

It could also mean that patients with this mutation do not develop cancer, but have a greater involvement of brain, such as Alzheimer disease.

It is possible to speculate 2 WS variants: 1) cancer-related WS; 2) degenerative neurologic disease related WS.

The 2 conditions could be mutually exclusive of each other.

The features found are in line with the general tendency for premature aging in Werner syndrome. In fact, it is clear and demonstrated that normal aging people shows physiological nerve changes like as moderate fiber loss, slight axonopathy and segmental de-myelination with some attempt of re-myelination.

Furthermore, the weak tendon reflexes found here could be due to the simultaneous presence of peripheral neuropathy.

These findings suggest that diffused structural and metabolic tissue damage can be present, in brains of WS patients.

Brain changes can be part of an accelerated aging process indeed that, as in many organs, may extend to CNS or can, perhaps, be related to more complex pathogenetic mechanisms such as apoptosis (Battisti C et al 2000).

These data are the clue that neurophysiologic impairment is possibly more frequent than generally believed, probably because of it generally is not evaluated.

These two patients were followed over time by doctors and specialists. There have been regularly examined the values and parameters related to metabolic dysfunctions.

From diagnosis to date no type of cancer was detected. This could be due to the type of *WRN* mutation that does not lead to metabolic changes so important for the development of cancer.

They may also possess an unknown and complex “genetic background” that can protect them.

Their lifestyle or the environmental factors to which they are subjected may be not so critical to the development of other malignancies.

Or the actual cause may simply be that these individuals are still quite young (average age for the onset of tumors in WS patients is about 40 years) and they could develop cancers in the next years.

It is very important to continue to follow these patients over time, so that if they were going to develop some kind of tumor, this can be detected in its early stage.

Neurological complications in Werner syndrome

In Werner syndrome neurological complications are usually regarded as uncommon, but they may be under-recognized.

Some of these, apart from muscle wasting, have been commonly reported in patients with Werner syndrome, but they may be more prevalent than previously realized.

There are described WS patients with sensory peripheral neuropathies or myelopathies. One third of the patients in whom the neurological findings were reported had loss of distal tendon reflex.

Evidence from the literature suggests that the peripheral neuropathy reported in association with WS is usually mild and mainly affects sensory nerves (Anderson and Haas 2003).

Moreover, the possibility of a coincidental association between Werner syndrome and peripheral neuropathy and myelopathy cannot be excluded.

However, the neurological symptoms and signs in these patients are typically mild and it is likely that these disorders have been under-recognized in the past.

The relationship between cognitive impairment and Werner syndrome is uncertain. Dementia has been reported in only few patients (Murata and Nakashima 1982).

Amyloid plaques and neurofibrillary tangles have been detected in other WS patients, but these abnormalities were insufficient to diagnose Alzheimer disease (Leverenz et al. 1998).

Moreover, the *WRN* C1367R variant was studied to investigate its association with Alzheimer disease, but the results supported the idea that this polymorphism was not involved as a risk factor for developing that degenerative disease (Payao et al. 2004).

Results of the research among elderly patients at a geriatric institute in Milan

Finally, another unique opportunity was the possibility of analyzing the database of an important Geriatric Institute concerning patients who were admitted during the last 10 years. In fact, Pio Albergo Trivulzio (PAT), a Geriatric Hospital in Milan, Italy, is the larger geriatric institute in Europe, that can be considered the ideal location to test how many individuals with Werner syndrome or related syndromes, are present among subjects who require admission to nursing homes, or to a geriatric institute, because of rehabilitation for senescence related diseases or alterations, i.e. pressure ulcers, diabetes and/or diabetic foot, brain degenerative diseases and/or other aging or premature aging related diseases.

Unfortunately, among all the individuals admitted to this geriatric hospital, was not found any patients with Werner syndrome.

This finding was not able to add additional information to our study, if not the fact to prove once again that Werner syndrome, like as other progeroid syndromes, is extremely rare.

At the same time it was possible to compare the incidence of degenerative diseases of the central nervous system (CNS), such as Alzheimer's (AD), among all patients in this Reference Centre, and in particular the relative rare incidence of different types of neoplasms in these degenerative disorders.

12. Alzheimer disease and its impact on the elderly people

Alzheimer disease (AD) is characterized by dementia that typically begins with subtle and poorly recognized failure of memory, also confusion, poor judgment, language disturbance and together these conditions slowly become more severe and, eventually, incapacitating.

It is due to a degenerative process consisting of loss of neurons in specific brain areas, the presence of neuritic plaques and degeneration of fibrillary.

Death usually results from general inanition, malnutrition, and pneumonia. The typical clinical duration of the disease is eight to ten years, with a range from 1 to 25 years.

Approximately 25% of all AD is familial of which approximately 95% is late-onset (age >60-65 years) and 5% is early-onset (age <65 years).

The distinction between early-onset familial Alzheimer disease and late-onset familial Alzheimer disease is somewhat arbitrary.

AD is the most common cause of dementia in North America and Europe, with an estimate of four million affected individuals in the US.

The prevalence of AD increases with age:

- Approximately 10% of persons over age 70 years have significant memory loss and more than half of these individuals have AD.
- An estimated 25%-45% of persons over age 85 years have dementia.

The incidence of AD rises from 2.8 per 1,000 person years in the 65-69 year age group to 56.1 per 1,000 person years in the older than 90 year age group (Kukull et al 2002).

Establishing the diagnosis of Alzheimer disease relies on clinical-neuropathologic assessment. Neuropathologic findings of β -amyloid plaques and intraneuronal neurofibrillary tangles remain the gold standard for diagnosis.

The clinical diagnosis of AD, based on signs of slowly progressive dementia and findings of gross cerebral cortical atrophy on neuro-imaging, is correct approximately 80%-90% of the time. The association of the *APOE* e4 allele with AD is significant, though neither fully specific nor sensitive; moreover three forms of early-onset familial AD caused by mutations in one of three genes (*APP*, *PSEN1*, *PSEN2*) are recognized.

Individuals with non-familial AD, this disease may occur anytime in adulthood.

Its exact pathogenesis is still unknown. A common hypothesis is that non-familial AD is multifactorial and results from a combination of aging, genetic predisposition, and exposure to one or more other environmental agents including head trauma, low education level, viruses, and/or toxins, although no environmental agents have been proven to be directly involved in the pathogenesis of AD.

However, it is often speculated that late-onset AD is the result of unknown environmental factors acting on a predisposing genetic background (Borenstein et al 2006).

Treatment is supportive; each symptom is managed on an individual basis; assisted living arrangements or care in a nursing home is usually necessary; although the exact biochemical basis of AD is not well understood, it is known that deficiencies of the brain cholinergic system and of other neurotransmitters are present.

Drugs that increase cholinergic activity by inhibiting acetylcholinesterase produce a modest but useful behavioral or cognitive benefit in a minority of affected individuals.

Treatment trials evaluating use of anti-inflammatory agents (NSAIDs), estrogens, nerve growth factors, ginkgo biloba, statins, BACE inhibitors, and antioxidants are under way or recently reviewed (Klafki et al 2006, Masters & Beyreuther 2006).

Alzheimer's disease is a progressive disease, which requires long-term admission to hospital, nursing homes or rehabilitation hospitals, as well as described above.

At the Pio Albergo Trivulzio (PAT) Geriatric Hospital, a partly retrospective (2004-2007) and partly prospective analysis (2008-2009) was performed to detect among subjects hospitalized for Alzheimer's disease during years 2004-2009, the incidence and/or prevalence of those with concomitant (previous or current) clinically evident malignancies. In particular, the geriatric hospital has 79 beds for acute rehabilitation of Alzheimer patients and 75 beds for long-term admission of Alzheimer subjects.

During 2009, 420 different AD subjects are admitted to the hospital (220 admitted to the Dep. of Acute Rehabilitation and 75 to long-term hospitalization, "nursing home", with a presumed diagnosis of Alzheimer's disease), whereas 1280 had consultation and were followed-up as non institutionalizing patients.

Present data concern the prevalence of cancer in an overall population of 1083 subjects who were admitted to the Dep. of Acute Rehabilitation for Alzheimer's disease during years 2004-2009 (mean admission rate for year = 240 patients).

Concerning the prospective part of the study, in these 5 years were identified totally 79 cases with both Alzheimer and cancer, in particular 27 patients were diagnosed during years 2008-2009.

Only 5 out of them died of cancer during these 2 years.

The site and type of cancer in subjects with Alzheimer's disease and previous or concomitant malignancy are reported on Table 4.

Of these 79 patients, 51 were females and 28 males, mean age 83 years (range between 55 and 96) and mean age at diagnosis of malignancy was 77 years. The ratio of AD patients with cancer and others without cancer is 79/1083, i.e. 7,3%.

The overall prevalence of malignant diseases and the type of tumors didn't differ significantly from the prevalence of tumors occurring in old subjects of similar age in the general population.

Cancer of the breast, colon-rectum, stomach, liver and bladder were those more frequently observed (Tab. 15); these are the most common types of tumors occurring in the general population, in particular occurring in the adult phase of the life, not particular different from AD patients group.

Actually, 15 patients with breast cancer and with colorectal cancer had initial diagnosis more than 10 years before hospital admission for AD and could be considered disease free and "cured".

But, anyway, there are some differences with the general population.

In particular, even if the type of cancer here reported are substantially similar or identical to those that were found in not AD elderly people, it is noteworthy that in the present series there was a lower than expected incidence of lung cancer (n= 2 out of 79, i.e. 2,5%) and moreover no patient with pancreatic adenocarcinoma or gallbladder carcinoma, despite their increased prevalence among old subjects (ranking respectively 3th and 5th in the list of the most frequent tumors of the digestive tract).

Whether this absence is due to chance, the small number of incident cases or the usual short survival after diagnosis (<12 months) of patients with these two types of tumors, or to the actual decreased incidence of pancreatic cancer in subjects with Alzheimer's disease cannot be ascertained on the basis of present data and numbers.

Anyway, the mean survival after initial diagnosis of malignancy (6 years, i.e. 77-83y) suggests that Alzheimer's disease is not a condition which seems to accelerate premature death in subjects with previous or current diagnosis of cancer.

Table 15: Concomitant or previous malignancies in Alzheimer subjects

Site of tumor	HISTOTYPE	N	Males	Females
Breast cancer	Infiltrating Ductal	24		24
	Lobular	1		1
Colon	Adenocarcinoma	10		
Rectum	Adenocarcinoma	3		
Colon-rectum + bladder	Adenocarcinoma	2		
Prostate	Adenocarcinoma	9	9	
Stomach	Adenocarcinoma	5		
Liver Liver metastases	Hepatocarcinoma	3		
	Unknown	1		
Bladder		3		
Lung	Squamous cell	1		
	Small cell	1		
SNC	meningioma	1		
Esophagus, urothelium, anus, uterus, ovary, tongue, Kaposi's, lymphoid leukemia, hairy cell leukemia		1 each		
Miscellaneous		6		

Total Number **79**

Original data were obtained from 1) the personal observation of 2 patients with Werner syndrome, who were admitted to the University Hospital of Siena, but without the development of any type of neoplasm, 2) the access to the database of the of the largest Geriatric Institute in Europe in the search of patients with WS, who could be followed because of some and different complications of the disease.

But it was possible also to analyze 2 unique series, the former including malignancies associated with institutionalized patients with Alzheimer disease, and the latter, focusing on associated diseases, including cancer in one case, in 13 ultracentenarians, all females (aged 100 to 108 years) admitted to the PAT institute (Fig. 22).

Geriatric resource	centenarian individuals	(%)	Geriatric resource	centenarian individuals	(%)
USA	32 (110-119y)		PAT (Italy)	13 (100-108y)	
Female	27		Female	13	
Male	5		Male	0	
Cataract	28	88	Cataract	11	85
Osteoporosis	14	44	Osteoporosis	6	45
Hypertension	7	22	Hypertension	4	30
Stroke	4	13	Stroke	2	15
Myocardialinfarction	2	6	Myocardialinfarct	2	15
Parkinson	0		Parkinson	1	8
Diabetes	1	3	Diabetes	2	15
Cancer	8	25	Cancer	1	8
colon cancer	2		colon cancer	1	
breast cancer	2		breast cancer	0	
skin cancer	2		skin cancer	0	
bladder cancer	1		bladder cancer	0	
unknown cancer	1		unknown cancer	0	

Fig. 22: Secondary pathologies observed among ultracentenarians in Italy (PAT) and in United States. Only one patient of Pio Albergo Trivulzio has shown a tumor, in particular a colon cancer.

Comment

A concerning inverse association between Alzheimer disease and cancer prevalence

The linkage between cancer and Alzheimer's diseases has been regarded as a "diminished cognitive reverse" which could be a mechanism that increases the likelihood that patients with cancer may be later diagnosed with other neurologic diseases, including Alzheimer disease.

Possible explanation is that a cognitive dysfunction, sometimes already present in old people with cancer before treatment, may worsen acutely secondary to treatment-related neurotoxicity, and may continue after cessation of therapy (women with breast cancer, men with prostate cancer).

On the other hand, a recent investigation by Roe (2005) using a prospective longitudinal design, including comprehensive neuropsychological assessment of cognitive function and histopathologic determination of dementia subtype, not only failed to find increased risk of developing dementia in patients with a history of cancer compared with cancer-free participants, but also reported a statistically non significant trend suggesting that the risk of developing dementia of the Alzheimer's type was actually marginally less in patient with a prior history of cancer than in cancer-free participants.

These studies suggest that the presence of AD (hazard ratio 0,41) and pure AD (hazard ratio 0,31) was associated with a reduced risk of future cancer hospitalization, whereas no significant association was found between cancer and subsequent development of vascular dementia.

Since prevalent AD was associated with a 60% reduced risk of incident cancer (i.e. the risk was double in non AD), about 360 cancer cases should have been observed among the 2107 non AD persons (15%), and only about 12 among the 165 with AD (7%).

Twelve (or a similar number) incident cancers is not a proper number to try to disentangle the prevalence of the various sites or types of incident malignancy.

Other studies (Behrens et al. 2009; Staropoli JF 2008) showed an inverse association between AD and cancer, such that the rate of developing cancer in general with time was significantly slower in participants with AD, while participants with a history of cancer had a slower rate of developing AD.

However, the type of cancer associated or not associated (mutually exclusive) with AD is not a trivial information, and we would suggest caution before making inferences concerning disruption of basic biological or genetic mechanisms (input to die or survive), (Behrens 2009).

In cancer, cell regulation mechanisms are disrupted with augmentation of cell survival and/or proliferation, whereas conversely, AD is associated with increased neuronal death, either caused by, or concomitant with beta amyloid (A β) and tau deposition.

Future research may lead clues about the nature of both AD and cancer.

In particular, a putative common biological mechanism that inversely operates in the 2 disorders, one leading to increased cell growth or survival, and the other to a higher risk of cell death, could explain these results.

Genetic polymorphisms, DNA methylation or other mechanisms that induce changes in activity of molecules with key roles in determining the decision to “repair and live” or “die” could be involved in the pathogenesis of the 2 disorders.

The Authors hypothesize the possible role of p53, PIN1, and the Wnt signaling pathways that can be differentially activated or disrupted in the 2 diseases and could represent potential candidates that, speculatively, may explain inverse association between AD and cancer. (Behrens 2009).

We agree that further efforts should be made to investigate whether a protective relationship exists, or a common mechanism underlies this intriguing relationship, i.e. that excessive apoptosis seems to be associated with AD, whereas too little apoptosis may be associated with cancer.

However, cancer is not a single entity, and does not occur according to just one mechanism, such as simple cell proliferation.

Obviously, uncontrolled cell proliferation facilitates cancer occurrence.

In particular, there are some cancers, which are more strictly related to dietary habits or ingestion of foods, others to inhaled xenobiotics (asbestos, particulate material, transitional metals), others to radiation (thyroid cancer, leukaemia) or even to UV exposure or sun light (melanoma, skin cancer).

Excessive simplification or oversimplification of such a complex question involving gene-gene interaction, and gene-environment interaction, but also post transcriptional modulation can be misleading and is contrary to everyday clinical experience.

Tumorigenesis is a complex phenomenon and most of basic questions are still unanswered.

This question is not between AD and glioblastoma, i.e. a degenerative or proliferative disease in the same tissue, but if in this disease there is a generic protection against whatever unspecified type of tumor.

In our opinion, after establishing that the inverse relationship between AD and “generic occurrence of cancer” has been suggested in the 1990 and then confirmed and replicated by multiple independent studies (some of which longitudinal studies overcoming previous limitations), despite the small numbers of incident and non incident cancer in AD subjects, instead of waiting for decades before more conclusive data from prospective studies, there is a major necessity to establish a more accurate speciation of the various types of cancers.

Only after establishing “which is which” (on the basis of a precise histological conformation of the type of tumors, along with a detailed classification of them not obtainable by questionnaires), for instance pancreatic cancer and glioblastoma were among those neutrally exclusive with AD, then and only then more accurate mechanistic speculations can be made and a proper biological and pathophysiological plausibility can be envisaged.

In conclusion, after initial documentation and subsequent confirmation of an inverse relationship between AD and some types of cancer (each related to various causative factors and different pathways), also hypothesizing that the causal linkage could not be a simple algebraic score among genes or polymorphism, but that more complex interactions could occur other non-genetic factor involving inflammation, immune response, sex related factors, hormone regulation etc.

Discussion

The present thesis includes data deriving from very different and not easily comparable sources and series. Therefore, no clear-cut cause and effect relationship can be inferred from genotype - phenotype correlations concerning the causative role of a given germ-line mutation in the *WRN* gene and the occurrence of an increased incidence of cancer or, more specifically, the occurrence of a peculiar type of neoplasm in an affected subject.

One obvious limitation was the small number of subjects with clinically evident Werner syndrome and associated cancer, who had detection of the precise site of their germ-line mutations.

Therefore, the absence of statistically significant genotype-phenotype correlations could be alternatively due to the too small number of informative cases or to the lack of a precise correlation.

A clear correlation has been detected in well known multitumoral syndromes caused by germ-line mutations of tumor suppressor genes, such as Familial Adenomatous Polyposis (FAP, mainly caused by germ-line mutations of the *APC* gene, mapped at 5q21) or *MEN2A*, caused by germ-line mutations of the *RET* gene, mapped at 10q11).

In particular, concerning FAP, a clear-cut association has been found between the site of germ-line mutation of the causative gene and the type and number of colonic polyps, the prevalence of associated non tumoral findings such as CHRPE, or the incidence of extracolonic alterations, such as papillary thyroid carcinoma (PTC), hepatoblastoma (HB) or brain tumors (BT), even if other genetic, epigenetic, or environmental factors (mainly sex related factors) should also cooperate for the phenotypic occurrence of these manifestations (Cetta et al 1997 – 2010).

Concerning *MEN2*, most surgeons advocate that the ideal age for prophylactic surgery for medullary thyroid carcinoma (MTC) for patients carrying a mutation in exon 11, codon 634, is as early as age 5 (Schellhaas E et al 2009).

DNA analysis used to identify risk for *MEN2B* have shown germ-line mutation in codon 928 of the *RET* protooncogene. In these patients MTC tends to present at a very early age and is multifocal, bilateral and more aggressive (Seni T et al. 2008). In addition, *RET* mutations, more frequently associated with MTC were found at codons 609, 611, 618, 620, 634, but no statistical evidence that the presence of persistent or recurrent medullary thyroid carcinoma was dependent on the specific genetic alterations (Skinner MA et al. 2005).

Original data were obtained from 1) the personal observation of 2 patients with Werner syndrome, who were admitted to the University Hospital of Siena, with no evident tumor, 2) the access to the database of the of the largest Geriatric Institute in Europe (Pio Albergo Trivulzio - PAT - , Milan, Italy) in the search of patients with WS, who could be admitted to a geriatric hospital because of complications of the WS, but also to analyze 2 unique series, the former including malignancies associated with institutionalized patients with Alzheimer disease, and the latter, focusing on associated diseases, including cancer in one case, in 13 ultracentenarians, all females (aged 100 to 108 years) admitted to the PAT institute.

Finally, an exhaustive literature research has been made, in the search of all reported cases of WS, both as single case report and as series or cohort of patients, either as single clinical series or in association with the specific germ-line mutation in the *WRN* gene.

Obviously, the main focus was on subjects with 1) Werner syndrome, 2) associated neoplasms; 3) germ-line mutation of the *WRN* gene.

Unfortunately, only 23 of these patients could be found.

Therefore, available data came from different series and sources, and discussion and inferences will be jeopardized by this lack of homogeneity.

However, we deem that some interesting observations and speculations can be made even on the basis of this inhomogeneous but, for other aspects, unique collection of data, both original and derived from the literature.

Analogously to the observation of ultra centenarians with cataract and osteoporosis but with a very low incidence of previous or associated tumors, it is plausible that early senescence, with a very high incidence of cataract (100%) and osteoporosis (94%), doesn't mean an increased incidence of all types of neoplasms, as an obliged associated finding, simply on the basis of the equation that, since tumors are more frequent in the elderly, earlier senescence must be associated to an increased incidence of tumors typical of senescence at an earlier age, i.e. anticipated of 2 or 3 decades.

Even stressing once again that the number of informative cases is too small to draw definitive conclusion, the overall amount of information included in the present study, however, supports the view that: 1) patients with Werner syndrome and their usual complications are not commonly present in geriatric hospitals; 2) epithelial cancers most frequently present in the general population are not anticipated of 2 – 3 decades in patients with Werner syndrome, 3) early senescence and/or some typical features of senescence such as cataracts and osteoporosis are not necessarily associated with an increased incidence of neoplasms.

On the contrary, it can be hypothesized that Werner syndrome concerning the supposed association with an increased and almost obliged prevalence of tumors, approaching WS to a typical inherited multitumoral syndrome, is a heterogeneous syndrome.

This is in accordance with the previously reported 41% of association with cancer.

In particular, it can be suggested that, whereas there are some germ-line mutations which are undoubtedly associated with an increased prevalence of neoplasms, - some of which are very peculiar and likely associated with the disrupted function of the mutated protein, - there are some other germ-line mutations which don't show this cancer susceptibility. Interestingly, some of these patients, analogously to what seems to occur in patients with Alzheimer or Parkinson diseases (neurodegenerative diseases) could also show an inverse association with cancer occurrence.

Namely, the 2 patients with WS observed in our University Hospital didn't show any sign of cancer, despite the fact that the age of both (52 and 42 years) is greater than the mean age (41, 2 years) of WS patients reported in the literature in association with already diagnosed cancer.

In particular, both of them had instrumental features of neurological changes, which are not usually found in typical WS patients.

This, on the basis of the supposed inverse relationship between degenerative CNS lesions and cancer, may suggest a "protective role" of "neurodegenerative alterations" in cancer occurrence.

Therefore, even if we don't know whether subjects with the phenotypic picture observed in our 2 patients with WS actually will show a decreased susceptibility to cancer (we should follow-up a consistent number of such patients up to the 80ths, but they usually die earlier, because of arteriosclerotic lesions), we can suggest a word of caution before stating that WS can be "pooled" together with other "inherited multitumoral syndromes", or that subjects with WS, even if they don't die because of vascular diseases, they will die of

malignancies, simply because malignancies are anticipated of 2 to 3 decades in this cohort of subjects.

In particular, analogously to what has already been suggested concerning the possible dual behavior of mutations in the *WRN* gene regarding the risk of arteriosclerosis and subsequent occurrence of myocardial infarction, the possibility is suggested (Fig. 23) that different sites of germ-line mutation of *WRN* gene could also affect the risk of cancerous transformation, leaving in the former case to an increased occurrence of malignancies, and in the latter to a normal or reduced risk of malignancy in comparison with the population of the same age showing the wild type *WRN* gene.

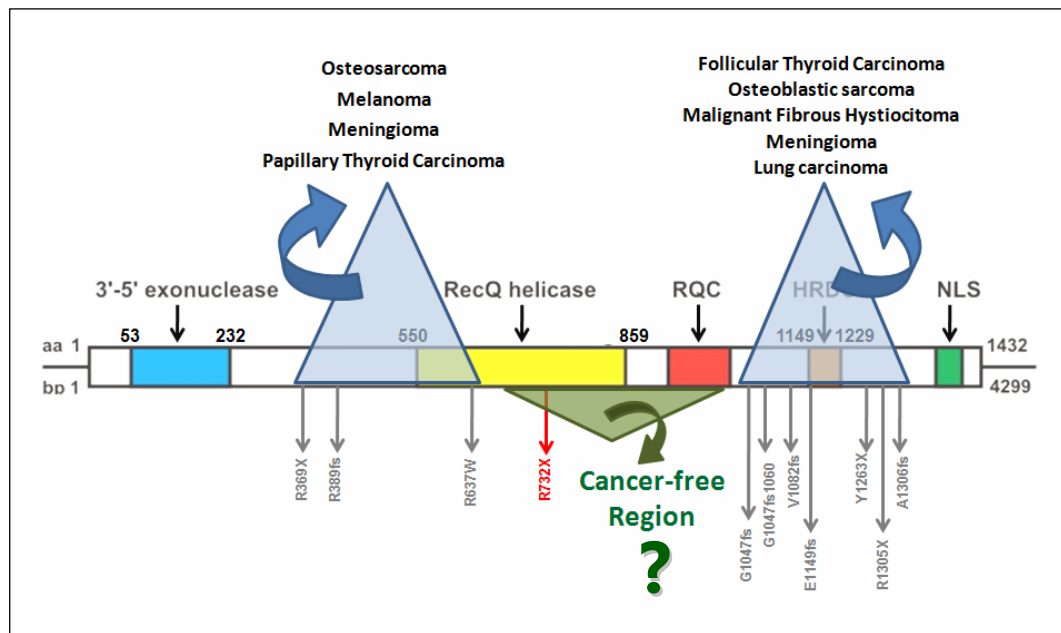


Fig. 23: Diagram that shows two positive spikes which correspond to the two high spot regions into the genomic areas, the former close to 5' and latter close to 3' end point, and one intermediate region (between codon 637 and codon 1047) in which the slope of the curve is oriented below the hypothetical basal line. This slope suggests that, in the absence of more detailed information, subjects with these mutations could have a reduced susceptibility to the cancers, in comparison to the wild-type. (See discussion for further information).

Concerning the type of associated tumors – in the 41% of subjects with WS, in whom neoplasms are part of the syndrome – some interesting observations and comments can be made, even with the obvious limitations of the small number of informative patients.

In this respect, we tried to use all available information even if we kept separate data in the overall population with clinically reported tumors from those in the subgroup with associated germ-line mutations ($n = 23$) and the general group of subjects with WS, including both those with associated and without associated neoplasms.

Pooling together all information available, but also keeping separate the various subgroups, some findings seem to be well documented:

1) the increased (abnormally increased) incidence of tumors of mesenchymal origin, namely osteosarcomas in WS patients; 2) the increased incidence of multiple tumors of different origin (epithelial and mesenchymal) in those with tumor occurrence; 3) the clustering of germ-line mutations most frequently associated with neoplasms in 2 segments of the *WRN*

gene, namely the area between codons 369 and 637 (close to the N-terminal), and the area between codons 1047 and 1306 (close to the C-terminal).

It must be outlined that the site of the germ-line mutations of the *WRN* gene in subjects not associated with neoplasms was the intermediate genomic area around codon 732 (see also other germ-line mutations from the literature).

Obviously, for the moment no attempt can be made to relate phenotype to the presumed segment of the gene and to the type of the truncated protein and its functional significance, also because there is very little information concerning the possible function of the various gene segments and, in particular, of the segment for RecQ helicase or HRDC.

Anyway, despite the lack of specific genetic and genotype-phenotype correlations, some speculations can be inferred, in particular from a pathophysiologic point of view.

Interestingly, osteosarcomas in WS patients (at lower extremities) occur in sites different from sporadic osteosarcomas (at upper extremities) and are associated with calcifications of soft tissues. These observations point to a common alteration in the osteogenic pathway, probably involving a main functional disruption, strictly related with the germ-line mutation, also determining scleroderma, and other mesenchymal alterations.

The other striking finding is the high reported incidence of follicular carcinoma of the thyroid (FTC). This is also an unusual feature, because other multitumoral syndromes were associated with an increased incidence of papillary thyroid carcinoma (PTC), such as FAP subjects, or with medullary thyroid carcinoma (MTC), such as MEN2 subjects.

Papillary and medullary thyroid carcinomas derive from two different types of cells (follicular cells and calcitonin secreting parafollicular C cells), but the unusual increased incidence of follicular cancer in subjects with WS deserves accurate evaluation, because it could give the clue for a better knowledge of thyroid carcinogenesis.

In fact, rare syndromes and inherited multitumoral syndromes often give useful information for a deeper insight into molecular pathways and mechanisms of more common sporadic malignancies.

Again, it must be outlined that the same germ-line mutation, in particular the c.3139-1G>C; p.1047fsX1061 alteration (mutation 4), was that most frequently associated with osteoblastoma and follicular thyroid carcinoma.

Interestingly, there was a different gender prevalence, namely 5 M and 3 F in the patients with osteosarcoma and, at the opposite, 5 F and 3 M in subjects with associated thyroid carcinoma.

This could be less surprisingly, simply because of the increased incidence of thyroid malignancies in the female sex (F:M rates 3:1 in sporadic tumors; 50:1 in FAP associated PTC).

The third homogeneous group of tumors that resulted particularly frequent in WS patients was that of meningiomas (n=33 in the overall literature, even if only 2 of them had precise detection of the germ-line mutation: 1105C>T and 3139-1G>C, respectively).

It is noteworthy that in both cases, they were associated with thyroid carcinoma.

Interestingly, contrary to what occurs in FAP subjects who, despite having a similar gender incidence of colonic polyps and cancers (M:F ratio = 1), show a striking female prevalence in other extracolonic manifestations (PTC, BT), subjects with WS, who don't show any gender prevalence for the occurrence of the syndrome, concerning some unusually associated tumors, namely meningiomas, osteosarcomas, but also melanoma, show an increased male prevalence.

Therefore, considering the overall amount of available data concerning associated tumors, it seems that unusual tumors of mesenchymal origin either develop at an earlier age, such as

meningiomas (anticipation of 2-3 decades in comparison with sporadic tumors), or occur at usual age (mostly during the first 2-4 decades) as sporadic tumors but with an increasing incidence.

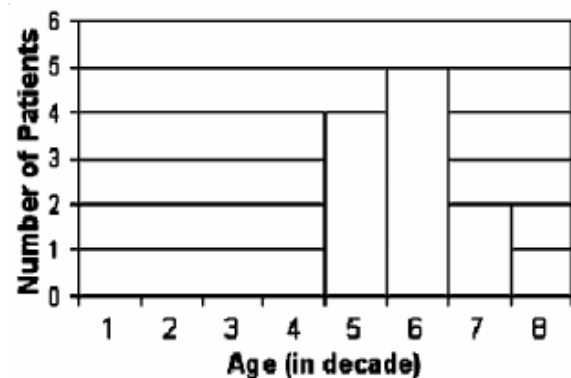
It remains more difficult to explain, for the moment, and to relate to the common disruption of pathways of mesenchymal development, the increased and unusual incidence of follicular thyroid carcinoma.

A more accurate histological and molecular examination of these specimens, in particular in comparison with other associated abnormalities, could facilitate a deeper insight into probable pathogenetic linkages.

The other finding that is noteworthy in subjects with Werner syndrome is the unusually low incidence of the most frequent epithelial cancers, which are those prevalent in sporadic cases.

There is a striking lack of tumors of the lung, colon, breast, liver, bile tract, prostate, which are the most prevalent tumors in the general population.

One possible explanation could be that these neoplasms usually occur mainly after the age of 50 and most of subjects with WS don't reach the sixth decade of life, even if WS subjects older than 70 years are increasingly reported (Fig. 24).



(Fig. 24: distribution of the ages of death in a previous more extensive study; Huang et al. 2006).

However, data from cancer incidence in subjects with Alzheimer and/or Parkinson's disease, who usually live till old age and, more importantly, data from ultracentenarians, suggest that degenerative disease, namely cataract and osteoporosis, but also others pathologies may be mutually exclusive with cancerous or hyperproliferative diseases.

Therefore, caution is suggested before premature inferences concerning ageing and/or premature ageing and increased occurrence of neoplasms, because it is plausible that different populations are present, 1) one with increased susceptibility to cancer, both at early age (subjects with inherited multitumoral syndromes) and later, with increasing incidence with age, 2) another population, likely smaller, that can reach old age and even very old age (> 100 years) in the absence of cancer and 3) that people developing degenerative or chronic diseases may be "protected" from developing osteosarcomas, meningiomas and probably follicular thyroid carcinoma.

However, there could be other subjects with WS, who don't show an increased susceptibility to develop cancer.

Conclusions

In conclusion, the present study has many limitations and drawbacks, but also has some strengths.

Limitations include the small number of informative patients, that is too small to obtain statistically significant genotype-phenotype correlations, able to relate a given germ-line mutation of the *WRN* gene to a definite phenotype and/or to an increased incidence of neoplasms, also including the site or the type of the presumed malignancy, and then to be useful for clinical and therapeutical purposes, analogously to what has been suggested in other “multitumoral” syndromes.

Anyway, despite small numbers, some interesting correlations could be showed concerning 3 enough homogeneous groups of patients, namely those with follicular thyroid carcinoma, osteosarcomas and meningiomas, which tend to occur in association with some germ-line mutations more than with others.

Additional cases are required for a deeper insight into genotype-phenotype correlations. Other limitations include the long-term follow-up of patients with WS, either in case of associated germ-line mutations or in case of simple observational clinical studies.

In fact, even if data are reported concerning the increase of the mean age of death up to 54 years, in patients with this disease, the occurrence of late onset diseases or disabilities are not always reported. Therefore, detailed information for the purpose of the present thesis, namely concerning the occurrence of tumors which usually develops after 50 or 60 years, is lacking.

More precise information will be available when, because of improved medical treatment and/or prevention, a greater number of patients with WS will reach older age.

On the contrary, strengths include the fact that the study has been performed by a team with a well documented long-term experience on genotype-phenotype correlation, concerning other inherited multitumoral syndromes (FAP, HNPCC, MEN), in particular regarding manifestations which are not only the peculiar or obliged malignancy, but also associated malignancies, which for full phenotypic occurrence, require the concomitant presence, in addition to the germ-line mutation, of additional epigenetic or environmental (sex related) factors, rendering phenotypic manifestations more complex and difficult to disentangle.

In particular, it is still extremely difficult to establish “which is which”, i.e. what’s the relative role of the “inherited genetic mutation”, conferring the congenital predisposition to develop neoplasms, and the role (that sometimes can be prevalent) of environmental or epigenetic determinants, which could play a major role on subjects with only a “generic predisposition” to develop tumors in sites or organs different from the typical target of the syndrome.

Finally, another unique opportunity was the possibility of analyzing the database of the main geriatric Institute in Europe, concerning patients who were admitted during the last 10 years.

Even if the access to this database added little to the primary aim of the thesis, i.e. genotype-phenotype correlation in subjects with Werner syndrome, because no patients with WS was found among all those individuals admitted to PAT between 2004 and 2009, priceless information were obtained from the analysis of patients with Alzheimer and associated cancers and from the demographic and medical data of ultracentenarians.

These data unequivocally show that 1) senescence is not necessarily associated with an increased incidence of malignancy in every case; 2) some degenerative diseases, such as

Parkinson and Alzheimer diseases, can be mutually exclusive with cancer, and play a protective role, by preventing or excluding cancer occurrence.

These data can contribute to open new avenues concerning the historical association between aging – or premature aging – and tumorigenesis.

As it is usual in biological contexts, reality is far more complex than it has previously been presumed.

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