Aging Is Accompanied by a Progressive Decrease of Expression of the WRN Gene in Human Blood Mononuclear Cells

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The WRN gene encodes DNA helicase participating in genome maintenance. We looked for associations of natural aging with expression and methylation of this gene in blood mononuclear cells and with its common polymorphisms. Analyses were performed in ethnically homogenous Polish Caucasians. The mean level of the WRN messenger RNA was significantly lower in long-living individuals than in young and middle-aged controls (p < .001 and p = .025, respectively). Analysis of the 361 bp WRN promoter CpG island showed that aging might be accompanied by a slight increase of its methylation status; however, it seems to be biologically insignificant. Finally, analysis of the WRN R834C, L1074F, and C1367R polymorphisms showed that the frequencies of the L1074F and C1367R polymorphisms were similar in all age groups tested, whereas the R834C polymorphism was absent from Polish Caucasians. We suggest that age-related decrease of the WRN expression but not its common genetic variants might contribute to human immunosenescence.

Key Words: Aging—Werner syndrome gene (WRN)—Polymorphism—Expression—Promoter methylation.

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AGING results from accumulation of stochastic damage to the genomic DNA and to key proteins and lipids, with its rate depending on multiple factors (1–3). Aging is not a disease per se; however, by limiting the ability to maintain homeostasis, it is a major risk factor for development of age-dependent diseases, such as cardiovascular disease (CVD), type 2 diabetes (type 2 diabetes mellitus [DM2]), cancer, and neurodegeneration. The occurrence of such diseases considerably complicates its course and shortens the length of life (4–6).

Currently, it is estimated that genetic factors account for 25%–30% of the regulatory agents of human aging (7–9). For example, certain polymorphic variants of genes encoding the proteins of insulin and insulin-like growth factor 1 (IGF-1) pathways, such as insulin and insulin-like growth factor 1 receptors, KLOTHO, and FOXO transcription factors, as well as of the gene encoding apolipoprotein E are significantly more common in oldest humans than in younger individuals (10–14). On the molecular level, aging is associated with signs of genomic instability, such as accumulation of DNA lesions (various adducts, modifications, and breaks) and an increased number of chromosomal aberrations and of micronuclei (15–17). This, in part, is a result of inefficient DNA repair (18,19). Therefore, genes encoding proteins involved in the maintenance of genomic stability form another group of genes that might play a role in the regulation of the aging rate. Such a notion is supported by the fact that many segmental precocious aging syndromes result from mutations in genes encoding such proteins (20).

The WRN (RECQL2) gene encodes WRN, a 1,432 amino acid protein. It possesses helicase and exonuclease activities (21,22) and plays an important role in DNA repair, transcription, replication, recombination, and in telomere maintenance (23–26). Mutations of the WRN gene are a genetic cause of Werner syndrome (27), a segmental progeroid disease most closely reminiscent of normal aging (28). Lack of C-terminal end containing nuclear localization signal results in retention of the mutated protein in the cytoplasm (29); this molecular phenomenon accounts for phenotypic uniformity among Werner syndrome patients (short stature, premature graying, skin atrophy, juvenile bilateral cataract, DM2, atherosclerosis mostly in arterioles, CVD, osteoporosis mostly in the long bones of legs, cancers with excess of soft tissues sarcomas and osteosarcomas, trophic ulcerations around the ankles and elbows, calcifications of cardiac valves, and finally, death due to CVD or cancer) regardless of type and localization of mutation (20,30).
These observations prompted us to check if certain functional polymorphisms of the WRN or its modified expression might be associated with longevity, possibly due to the enhancement of WRN efficiency as guardian of the genome. In this work, we present evidence that the R834C WRN polymorphism is absent from Polish Caucasians and that reaching 100 years of age is not associated with the L1074F and C1367R polymorphisms. We also show that aging is associated with decreased WRN expression in blood mononuclear cells (BMCs), which could contribute to immunosenescence (31,32).

**Materials and Methods**

**Study Subjects**

Analysis of the WRN genetic variants was performed in ethnically homogenous Polish Caucasians: 149 centenarians (C, 99.6–107.2 years old, mean age: 101.1 years, 130 women, and 19 men) (33) and 414 healthy blood donors and volunteers (Y, 18–45 years old, mean age: 27.9, 233 women, and 181 men). In addition, because precocious aging Werner syndrome patients have a very high prevalence of CVD and DM2, two additional control groups of the same ethnic background were studied: 226 patients who suffered from myocardial infarction (MI) before the age of 55 years (28–55 years old at first MI, mean age: 46.9 years at first MI, 61 women, and 165 men) and 190 patients diagnosed with DM2 before the age of 55 years (26–55 years old at diagnosis, mean age: 46.1 years at diagnosis, 120 women, and 70 men). Analysis of theWRN expression was performed in 20 participants from the Y group (22–37 years old, mean age: 28.5 years), in 18 healthy people of middle age (M, 60–69 years old, mean age: 65.7 years, participants of PolSenior study), and in 17 long-living individuals older than 90 years (LL, 90–100 years old, mean age: 92.8 years; because messenger RNAs [mRNAs] from only 5 centenarians were available, the LL group was formed from these individuals and from 12 nonagenarians). Analysis of the WRN promoter methylation was performed in 10 participants from the Y group (25–37 years old, mean age: 29.5 years) and in 10 participants from the C group (100–102.4 years old, mean age: 101.4 years) as well as in 10 additional participants that formed middle-aged control group of healthy individuals (M, 65.3–66.5 years old, mean age: 65.9 years, PolSenior). The study protocol was approved by the Ethical Committee of the Medical University of Warsaw. All participants gave a written informed consent for participation in the study.

**DNA Isolation and Analysis of Restriction Fragment Length Polymorphism**

Genomic DNA was isolated using salting-out procedure (34). Genotyping of the selected polymorphism in the WRN was done by polymerase chain reaction (PCR) amplification followed by digestion with an appropriate restriction enzyme (restriction fragment length polymorphism method [RFLP]). Analyses of the R834C and C1367R polymorphisms were performed according to previously described methods (35,36). The L1074F polymorphism was analyzed as follows: The 188 bp DNA fragment was amplified with the forward 5′AATGAGAAATTTGTGTCACAAAGGCT3′ (underlined is a mismatch introduced to generate restriction site) and reverse 5′ATCCCTTCCCCAACTGAC3′ primers in a PCR reaction performed with Taq polymerase (Invitrogen, Carlsbad, CA) in a buffer containing 2.5 mM MgCl₂. The PCR conditions were as follows: initial denaturation at 94°C for 4 minutes, 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and final elongation at 72°C for 5 minutes. RFLP analysis was performed with the HindIII enzyme. The restriction fragments were visualized on 2%–3% agarose gels. A 188-bp band represented the G allele, whereas 166- and 22-bp bands were associated with the presence of the T allele.

**Bisulfite Treatment of DNA and DNA Methylation Analysis**

Bisulfite modification of the genomic DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research Corporation, Orange, CA). Five hundred nanograms of each genomic DNA was bisulfite modified following the manufacturer’s protocol with the previously described modifications (37). Bisulfite-treated DNA was purified and resuspended in 10 μL of the M-elution buffer supplied by the manufacturer. Fifty nanograms (1 μL) of this DNA was used as a template in 12.5 μL PCR reaction performed with hot-start Platinum Taq polymerase (Invitrogen) and with sense 5′GGYGGGTATTATATATATTTTTG3′ and antisense 5′CCCCCTCTTCCCCCTCAAC3′ primers. The PCR conditions were initial denaturation at 94°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute, and final elongation at 72°C for 10 minutes. The PCR product of 361 bp was gel purified and cloned into the pGEM-T vector (Promega, Madison, WI). Three clones from each person were sequenced.

**Isolation of Blood Mononuclear Cells**

BMCs were isolated according to the method of Neitzel (38) with modifications (37).

**RNA Isolation, Reverse Transcription, and Real-Time PCR**

Isolation of total RNA was performed as described in Polosak and colleagues (37). One microliter of complementary DNA corresponding to 0.5 ng of total RNA was used as a template in real-time PCR. The reaction was performed in LightCycler 480 Instrument II (Roche, Mannheim, Germany) with the LightCycler 480 Sybr Green I Master kit (Roche, Mannheim, Germany) and with sense 5′GGGTTTTAGAGGTTAGAAGC3′ and antisense 5′AGATATTCTTCTCAGACTGCTTG3′ primers as well...

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as with control β-actin sense 5’CAGCCTGGGATATGCGAAGCT- 
TACA3’ and antisense 5’TTCTACAAGACGCCTGTTG- 
3’ primers. PCR conditions were as follows: initial incubation at 95°C for 10 minutes, 40 cycles of 95°C for 12 seconds, 60°C for 12 seconds, 72°C for 12 seconds, and then one melting curve cycle. The results obtained for the WRN gene were normalized against the results for the β-actin gene.

**Statistical Analysis**

The genotype distribution was analyzed assuming three models of inheritance: dominant, codominant, or recessive. Under each model, the odds ratio with 95% confidence interval and the p value for an association was calculated. Furthermore, the Hardy–Weinberg equilibrium was checked with the χ² test. These calculations were performed using the Web-Assotest program (available at: http://www.ekstroem .com/assotest/assotest.html). The differences in quantitative parameters between groups were assessed using the Student’s t/Mann–Whitney U test or Krushal–Wallis analysis of variance with the Statistica software package (StatSoft, Tulsa, OK). Normality of distribution and homogeneity of the variance were checked with the Shapiro–Wilk and Levene’s tests, respectively. For all tests, the level of significance was established at 0.05. To assess the statistical significance, two-sided test was used.

**RESULTS**

**The WRN mRNA Level in Blood Mononuclear Cells of Young, Middle-Aged, and of Long-Living Subjects**

The WRN mRNA level in BMCs was assessed using real-time PCR in 55 healthy study participants representing all allelic groups of the R834C, L1074F, and C1367R polymorphisms. Study participants were divided into three age groups. The results obtained for the WRN gene were normalized against the levels of the control β-actin mRNA. Real-time evaluation showed that the levels of the specific WRN mRNA varied markedly between cases within each age group: The difference ranged up to 7.8-fold in the Y, 10.3-fold in the M, and 9.0-fold in the LL group. Notably, the mean levels of the WRN mRNA were similar in men and women. When the mean level of the WRN mRNA in the Y group was normalized to 100% (±51.0% SD), the relative mean amounts of this mRNA were 77.3% ± 48.3% in the M group and 52.2% ± 35.4% in the LL group. The mean level of the specific mRNA was significantly lower in the LL group compared with the Y and M group (p < .0001 and p = .025, respectively). The difference between the Y and M was not significant (p = .332; Figure 1).

**Methylation Profile of the WRN Promoter**

To check if epigenetic drift might be responsible for the age-related decrease of the WRN expression, the methylation status of the 361-bp fragment of the WRN promoter, spanning from the −197 to the +164 position relative to the transcription start site, was assessed in 30 study participants. The investigated promoter fragment contained 46 potential methylation sites: 29 upstream and 17 downstream of the transcription start site. Sequencing of three clones from each individual (30 clones in each age group) showed that in all participants, the analyzed promoter fragment was sparsely methylated (Figure 2). In three Y, two M, and two C participants it was methylation free; in the remaining individuals, 1–4 of 46 potential sites were methylated. The number of methylated sites was similar in men and women. In total, of 460 potential methylation sites (100%) in each age group, methylation was present in 17 sites in the Y group, in 26 sites in the M group, and in 18 sites in the C group (Figure 2), which corresponds to 3.69%, 5.65%, and 3.91%, respectively. The differences between the Y and M, Y and C, as well as between the M and C age groups were not significant (p = .89, p = 1.0, and p = 1.0, respectively). Older age was associated with the increase of methylation at the sites located upstream of the transcription start site (290 potential sites [100%] in each age group). Thus, although in the Y group, two methylated sites within this promoter fragment were detected, in the M group, the methylation number rose to 18 and in the C group to 9, which corresponds to 0.69%, 6.21%, and 3.1%, respectively. The difference between the Y and M groups was significant (p = .032), whereas the difference
Y participants, most likely representing population distribution of polymorphisms of interest. No significant gender-dependent differences were detected.

The distribution of the \( WRN \) genotypes was then analyzed in the Y versus C age groups. No significant differences were observed between the studied groups (\( p = .799 \) and \( p = .980 \), respectively). Analysis of the Y versus the group consisting of centenarians never diagnosed with MI, DM2, or cancer (\( n = 126 \)) did not change this result (\( p = .844 \) and \( p = .963 \), respectively).

The distribution of the L1074F polymorphism was similar in the MI and Y groups (\( p = .177 \)), the MI and C groups (\( p = .366 \)), and in the MI and in centenarians never diagnosed with MI (\( n = 139, p = .417 \)). It was also similar in the DM2 and Y groups (\( p = .282 \)), the DM2 and C groups (\( p = .219 \)), and in the DM2 and in centenarians never diagnosed with DM2 (\( n = 143, p = .280 \)).

Similarly, no differences in the frequency of the C1367R polymorphism were detected between the MI and Y groups (\( p = .936 \)), the MI and C groups (\( p = .897 \)), the MI and centenarians never diagnosed with MI (\( p = .787 \)), the DM2 and Y groups (\( p = .766 \)), the DM2 and C groups (\( p = .818 \)),

Table 1. Distribution of the \( WRN \) Genotypes in Centenarians (C) and in Young Controls (Y)

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>C</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1074F</td>
<td>G/T</td>
<td>( n = 149 ) %</td>
<td>( n = 417 ) %</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>56</td>
<td>37.58</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>70</td>
<td>46.98</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>23</td>
<td>15.44</td>
</tr>
<tr>
<td>C1367R</td>
<td>C/T</td>
<td>( n = 149 ) %</td>
<td>( n = 414 ) %</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>13</td>
<td>8.72</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>59</td>
<td>39.6</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>77</td>
<td>51.68</td>
</tr>
<tr>
<td>R834C</td>
<td>C/T</td>
<td>( n = 148 ) %</td>
<td>( n = 417 ) %</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>13</td>
<td>8.72</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>59</td>
<td>39.6</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>77</td>
<td>51.68</td>
</tr>
</tbody>
</table>

*Note: \( n = \) number of participants.

Table 2. Distribution of the \( WRN \) Genotypes in MI and in DM2 patients

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>MI</th>
<th>DM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1074F</td>
<td>G/T</td>
<td>( n = 226 ) %</td>
<td>( n = 190 ) %</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>79</td>
<td>34.96</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>99</td>
<td>43.81</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>48</td>
<td>21.24</td>
</tr>
<tr>
<td>C1367R</td>
<td>C/T</td>
<td>( n = 226 ) %</td>
<td>( n = 190 ) %</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>17</td>
<td>7.52</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>93</td>
<td>41.51</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>116</td>
<td>51.53</td>
</tr>
<tr>
<td>R834C</td>
<td>C/T</td>
<td>( n = 200 ) %</td>
<td>( n = 190 ) %</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Notes: DM2 = type 2 diabetes mellitus; MI = myocardial infarction; \( n = \) number of participants.

*Not tested.

 Frequencies of the R834C, L1074F, and C1367R \( WRN \) Polymorphisms in Centenarians, Young Controls, and in Patients With Myocardial Infarction and With Type 2 Diabetes Mellitus

The frequencies of each \( WRN \) genotype in the tested groups are shown in Table 1 and in Table 2. Notably, in the studied populations, only the CC genotype of the R834C polymorphism was present.

The initial analysis was aimed at determining if the distribution of the L1074F and C1367R \( WRN \) genotypes differed in relation to gender. The analysis was performed in the
and the DM2 and centenarians never diagnosed with DM2 ($p = .871$).

**Discussion**

Genomic instability and telomere shortening are some of the hallmarks of cellular senescence (15–17). One of the reasons for the accumulation of lesions to the genomic DNA in aging cells might be the decreased efficiency of repair systems (18,39). The WRN encoding protein participating in the maintenance of genome integrity was previously indicated as potentially involved in the regulation of aging (40,41). It is suggested that long-living individuals (especially centenarians) form a group distinct from other humans who die earlier in terms of genetic and epigenetic factors regulating the rate of aging and longevity. To verify this hypothesis with respect to the WRN, we looked for associations between longevity and various potential alterations of this gene in individuals older than 90 years, including centenarians (when available), as well as in young and middle-aged study participants. Currently, 1 in approximately 1,000 Polish Caucasians is 93 years old or older, and only 1 in approximately 20,000 reached the age of 100; therefore, the chance that such longevity-predisposed persons were included in the Y or M groups was low. To further decrease such a probability, the WRN expression and methylation in these control age groups were analyzed in participants who did not come from long-living families.

Little information regarding age-dependent changes in expression of the WRN is available. It is known that its expression is decreased in in vitro aged fibroblasts (42). We examined the expression of the WRN on mRNA level in BMCs and found that it varied 8–to-10-fold within each age group we tested. There are at least two potential explanations for this phenomenon: heterogenic composition of the isolated BMCs samples or true individual differences in the expression of this gene. Indeed, BMC preparations could contain various proportions of diverse mononuclear cells, and each cell type could express the WRN mRNA to a different level, as shown by Bordi and coworkers (43). However, to be the sole reason for the expression variations observed by us within each age group, the content of our BMC preparations should be extremely distinct. To lower the chance of collecting BMC samples of such heterogeneity, we collected them from individuals with no signs or symptoms of any disease, including infection, at the time of blood drawing; nevertheless, heterogeneity of our samples might be, in part, responsible for the observed variations. We believe, though, that genuine individual differences in the WRN activity are mostly responsible for this phenomenon. Whether this is in fact the case needs to be determined. Our major finding is that aging is associated with a significant progressive decrease of the WRN expression in BMCs. To our knowledge, this is the first report describing such age-dependent changes in humans. Age-dependent gene expression alterations might be, at least in part, due to age-related changes in epigenetic signaling (44–47). When testing methylation of the WRN promoter, it was not our goal to describe any age-related differences in definite numbers. Instead, we wanted to see if methylation change occurs at all with aging; this is why we set the number of individuals in each age group at only 10 and the number of clones from each of them at 3. Sequencing showed that the mean methylation of the WRN promoter, mostly of its part located upstream to the transcription start site, was slightly higher in the BMCs of the M and C groups compared with those of the Y group. However, the statistical power of this finding is currently too low to draw a definite conclusion; but, based on these preliminary results, we hypothesize that methylation of the WRN promoter in BMCs might increase with age. This hypothesis should be verified by testing BMCs from a much larger number of individuals. In addition, age-dependent methylation of the WRN promoter might differ in various BMC subtypes; optimally, for in-depth analysis, BMCs should be first separated into subtypes. Another interesting observation was that methylation of the WRN seemed to be lower in the C than in the M group. This is consistent with findings of other authors claiming that centenarians form an exclusive group of people who, in terms of genetics and, possibly, epigenetics, differ from the rest of human population, and this uniqueness allows them to reach extreme longevity (48,49). Finally, we noticed that the pattern of methylation of the WRN promoter seemed to be random. Altogether, based on our results, we speculate that mostly other regulatory mechanisms, such as RNA interference or alterations in the expression of transcription factors, are responsible for a decreased expression of the WRN in human BMCs.

We also analyzed the R834C, L1074F, and C1367R polymorphisms of this gene in relation to age. It has been previously shown that the R834C variant polymorphism significantly reduces helicase and helicase-coupled exonuclease activity (35). Notably, we had reported that this polymorphism is not present in Caucasians of Polish origin. The L1074F variant and the C1367R variant polymorphisms only slightly change helicase activity relative to wild-type WRN (35,50). On the basis of a significant differences in their frequencies in healthy and ill study participants, these polymorphisms had been previously associated with certain life-shortening conditions. For example, L1074F was correlated with breast cancer (51), whereas the C1367R variant polymorphism was associated with protection against sarcomas and lymphomas (52,53), DM2 (54), as well as against CVD and myocardial infarction (55,56). Others saw no association between the C1367R and CVD (52). We found no association between being 100 years old or older and either L1074F or C1367R. Analysis of the statistical power of these tests showed that the risk of obtaining false-negative results was only approximately 10%. Our result regarding the C1367R polymorphism corroborates the
findings of Castro and colleagues, who analyzed it in Finnish population (57), and complemented the results of Kungingas and colleagues, who in their population-based Leiden 85+ Study found no association between these two polymorphisms and the aging trajectories and mortality (58). Therefore, we suggest that the analyzed polymorphic variants of the WRN influence neither the rate of aging nor the longevity. This notion is supported by our other findings showing no association between these polymorphisms and the risk of development of MI and of DM2, well-known age-related life-shortening diseases.

In conclusion, we have shown that neither L1074F nor C1367R polymorphism are associated with extreme longevity in Polish Caucasians. We have also shown for the first time that the amount of WRN mRNA decreases in human BMCs in an age-dependent manner and that the pattern of the WRN promoter methylation might slightly change with age. However, such methylation change seems to be of very little or of no functional significance. These observations suggest that age-related decrease in the WRN expression might contribute to human immunosenescence; however, the molecular mechanisms that underlie this phenomenon need to be elucidated.

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**References**