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Metabolic gene polymorphisms and *p53* mutations in healthy centenarians and younger controls

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To obtain insights into the genetic mechanisms of ageing, we studied the frequency of the simultaneous presence of polymorphisms in phase I and phase II genes and of several *p53* germline mutations in a group of 66 nonagenarians and centenarians in good health, selected from a larger sample of a multicentre Italian study in Northern Italy, and in a sample of 150 young healthy volunteers of the same ethnic group. We found a statistically significant difference in the frequency of the *GSTT1* deletion and the *p53* genotypes: the absence of any *p53* polymorphisms and of *GSTT1* deletion, and the simultaneous presence of the three *p53* polymorphisms and of *GSTT1* deletion, were much more frequent in young subjects than in centenarians (41.5% versus 26.9% and 8.8% versus 3.8%, respectively). One hypothesis to explain this difference is that subjects with both *GSTT1* deletion and *p53* polymorphisms may accumulate carcinogens and may have reduced DNA repair ability, and thus are more at risk for cancer. Another possible explanation is that both metabolic genes and *p53* act on pathways related to cell ageing and death, and therefore certain composite genetic patterns could represent a generic mechanism of protection against ageing, not just against the development of chronic diseases. It is likely that longevity is related to a complex genetic trait as well as to certain environmental exposures.

Keywords: ageing, molecular epidemiology, chronic diseases.

Introduction

Centenarians who have managed to reach the extreme limit of human life in good health are the best example of successful ageing. It is possible that they were able to escape the major diseases associated with age as a result of optimal environmental conditions and good lifestyle habits combined with some genetically determined resistance against age-associated diseases (Johnson 1997). Recently a possible antagonism between living to an old age and getting cancer has been suggested (Leroi *et al.* 2003).

Individual susceptibility to environmental carcinogens and toxicants is mediated by genetic differences. Many enzymes are involved in the metabolism of exogenous and endogenous carcinogens. These enzymes are the products of two major gene families: phase 1 (such as the *CYP* family) and phase 2 (such as the *GST*, *NAT* and *EH* families). Polymorphisms in these genes are very common in the general

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population (Garte *et al.* 2001) and are associated with various types of cancer (D'Errico *et al.* 1999). We have previously found a significant increase in homozygous deletion of the *GSTT1* gene in Italian nonagenarians compared with young healthy subjects (Taioli *et al.* 2001).

The homozygous *CYP1A1* Msp1 restriction fragment length polymorphism (RFLP) has been found to affect levels of expression of the gene (Garte *et al*. 2003), and has been shown to be associated with lung cancer in Europeans (Vineis *et al.* 2003). The heterozygote polymorphism has not been found to have any phenotypic effect.

p53 is a nuclear protein that plays a major role in tumour suppression via its involvement in multiple pathways, including apoptosis, cell cycle regulation and arrest, cellular transcriptional control and DNA repair. The *p53* gene is located on chromosome 17p13, and is one of the most commonly mutated genes in different types of human cancer (Hollstein *et al.* 1991). Besides the known example of germline mutations as the basis of the rare, familial Li-Fraumeni syndrome (Law *et al.* 1991), other more common polymorphisms have been found in this gene: in codon 72 in exon 4, a G→C transversion leads to an arginine (Arg) to proline (Pro) change (Miller *et al.* 2002); in intron 6 there is an Msp1 RFLP consisting of either six or eight variable bases (McDaniel *et al.* 1991), and a 16 bp duplication has been described in intron 3 (Lazar *et al.* 1993). These polymorphisms have been associated with lung cancer (Birgander *et al.* 1995, Biros *et al.* 2001), breast cancer (Själander *et al.* 1996, Wang-Gohrke *et al.* 2002), ovarian cancer (Wang-Gohrke *et al.* 1999) and bladder cancer (Biro *et al.* 2000). A previous study showed no differences in genotype frequency between healthy centenarians and controls (Bonafè *et al.* 1999). Two studies on lung cancer looked at the combined effect of both *p53* codon 72 polymorphism and *GSTs* polymorphisms. Both studies showed an increased risk of lung cancer when the combined genotypes were present (Liu *et al.* 2001, Miller *et al.* 2002).

Recently it has been demonstrated that the two *p53* variants at codon 72 are not functionally equivalent, either biochemically or biologically: they have different gene expression activities in inducing apoptosis and suppressing transformation, with the p53Arg variant more efficient than the p53Pro (Thomas *et al.* 1999).

The intronic polymorphisms *p53* Msp1 and the *p53* 16 bp duplication are situated in a non-coding region of the gene and do not seem to be functionally involved in the carcinogenesis process, but they do show strong linkage disequilibria with each other and with *p53* codon 72 polymorphism (Själander *et al.* 1995); for this reason it is very useful to study the combined genotype distribution for these three polymorphisms when analysing differences between groups, or associations with any type of endpoint.

We studied the frequency of the simultaneous presence of polymorphisms in phase I and phase II genes, and of three *p53* germline mutations, in a group of nonagenarians and a group of healthy young controls, to order to obtain insights into the genetic mechanisms of ageing.

Materials and methods

The study population comprised 66 nonagenarians and centenarians in good health, described in detail elsewhere (Franceschi *et al.* 2000), selected from a larger sample of a multicentre Italian study in Northern Italy on mechanisms responsible for successful ageing. The mean (±SD) age of the group was 100.1±2.2 years (range 95–105 years); 77% were females and 23% were males.

A sample of 150 young healthy volunteers of the same ethnic group as the centenarians were recruited in Northern Italy and used as a reference group. The mean (±SD) age of the control group was 42.5± 12.4 years (range 20–65 years). Since the majority of the centenarians were females, we used a population of female as controls.

All the subjects provided signed informed consent at the time of recruitment.

Genotyping analysis

Genomic DNA was extracted from peripheral blood lymphocytes and genotyping analyses were performed on all the subjects.

GSTM1 and *GSTT1* genotypes were determined in a multiplex polymerase chain reaction (PCR) analysis using primer pairs specific for *GSTM1* (5′-GAACTCCCTGAAAAGCTAAAGC-3′ and 5′-GTTGGGCTCAAATAT ACGTGG-3′) and *GSTT1* (5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′) together with a β-globin specific primer pair (5′-CAACTTCATCCACGTTCACC-3′ and 5′-GAAGAGCCAAGGACAGGTAC-3′). After an initial step of denaturation, 35 cycles were performed with denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and extension at 72°C for 1 min. Briefly, the absence (deleted) or presence of the *GSTM1*- and *GSTT1*-specific PCR products determined the respective genotypes, whereas the β-globin specific product acted as an internal control for a successful PCR.

*CYP1A1 Msp*1 (*CYP1A1*2)* genotyping analyses were performed using PCR as previously described (Taioli *et al.* 1995) using the following primers: 5′-TTAGGAGTCTTGTCTCATGCCT-3′ and 5′-CAGTGAAGAGGTGTAGCCGCT-3′.

The term 'wild type' refers to homozygotes for the common allele (absence of the Msp1 restriction site) and 'heterozygotes' refers to the presence of the polymorphism on one allele; when the polymorphism is present on both the alleles we used the term 'homozygotes'.

p53 polymorphisms were determined using the method of Själander *et al.* (1995). A set of primers, 5′-GCAGAGACCTGTGGGAAGCGA-3′ and 5′-ACCGTAGCTGCCCTGGTAGGT-3′, was used to analyse *p53* codon 72 and *p53* 16 bp duplication polymorphisms: 33–35 cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 65°C for 1 min 30 sec and extension at 72°C for 1.30 min. A fragment of 401 bp, or 417 bp if the 16 bp duplication is present, was amplified. To visualize the codon 72 polymorphism, 15 μl of PCR product was then digested with 8 U of BstU restriction enzyme and separated on 3% agarose gel stained with ethidium bromide. Two other primers, 5′-TATGAGCCGCCTGAGGTCTGG-3′ and 5′-TACAGGCA TGAGCCACTGCGC-3′ were used to study the Msp1 polymorphism; amplification conditions were similar to those previously described for the other *p53* polymorphisms, with an annealing temperature of 68°C for 1 min. PCR products were digested with 10 U of Msp1 and run on agarose gel.

The term 'wild type' refers to homozygotes for the common allele (presence of the BstU or Msp1 restriction site, and absence of the 16 bp duplication), 'heterozygotes' refers to the presence of the polymorphism on one allele, and 'homozygotes' referes to the presence of the polymorphism on both alleles.

Statistical analysis

Mean values and standard deviations were calculated for age.

Gene polymorphism frequencies are presented separately for centenarians and controls. Differences among gene frequencies were estimated using the χ^2 test or, when necessary, with the Monte Carlo simulation (Roff and Bentzen 1989). Values of $p \leq 0.05$ were considered to be significant.

Analysis of the data was performed using SAS 8.2 software system.

Results

We estimated the genotype frequencies of several genes involved in xenobiotic metabolism (*GSTM1*, *GSTT1* and *CYP1A1*2*) and of various *p53* polymorphisms (codon 72, Msp1 and a 16 bp duplication) for more than 95% of the study population (Table 1). No statistically significant differences in the genotype frequencies were found between the two groups analysed; *GSTT1* deletion was

Table 1. Frequencies of gene polymorphisms in centenarians and controls.

	Centenarians ($n = 66$)		Controls ($n = 150$)		
Gene polymorphism	*n*	%	*n*	%	*p* value
***p53* Msp1**					
Wild type (G/G)	42	63.6	101	67.8	
Heterozygous (G/A)	23	34.9	44	29.5	
Homozygous (A/A)	1	1.5	4	2.7	0.65
***p53* codon 72**					
Wild type (G/G)	33	50.0	81	54.3	
Heterozygous (G/C)	28	42.4	57	38.3	
Homozygous (C/C)	5	7.6	11	7.4	0.85
***p53* 16 bp duplication**					
Wild type	34	60.7	98	65.8	
Heterozygous	21	37.5	46	30.8	
Homozygous	1	1.8	5	3.4	0.59
CYP1A1*2					
Wild type (T/T)	46	73.0	124	82.7	
Heterozygous (T/C)	16	25.4	23	15.3	
Homozygous (C/C)	1	1.6	3	2.0	0.19
GSTM1					
Present	27	43.6	70	47.0	
Deleted	35	56.4	79	53.0	0.54
GSTT1					
Present	45	72.6	119	79.9	
Deleted	17	27.4	30	20.1	0.20

higher in centenarians compared with young subjects, as previously found, but the difference did not reach statistical significance.

In order to evaluate a possible interaction between *GSTT1* deletion and *p53* gene polymorphisms, we calculated the combined genotype frequencies in the two groups (Table 2). We found a statistically significant difference in the frequency of the *GSTT1* deletion and certain *p53* genotypes: the absence of any *p53* polymorphisms and of *GSTT1* deletion, and the simultaneous presence of the three *p53* polymorphisms and of the *GSTT1* deletion, were much more frequent in young subjects than in centenarians (41.5% versus 26.9% and 8.8% versus 3.8%, respectively). Combinations of *p53* genotypes and *GSTM1* or *CYP1A1*2* were not differently distributed in the two groups (data not shown). The absence of any *p53* polymorphisms and of both *GSTT1* and *GSTM1* deletion was 22.5% in controls and 13.5% in centenarians, while simultaneous presence of the three *p53* polymorphisms and of the double deletion of both *GSTT1* and *GSTM1* was 4.8% in controls and was not found in centenarians ($\chi^2 = 26.8$, $p = 0.04$).

Discussion

We studied the frequency of several gene polymorphisms in a population of centenarians in order to understand the mechanisms of ageing.

Table 2. Combined genotype frequencies in centenarians and healthy controls.

p53 Msp1	*p53* codon 72	*P53* 16 bp duplication	*GSTT1*	Centenarians ($n = 66$)		Controls ($n = 150$)	
				n	%	*n*	%
G/G	G/G	Wild type	Present	14	26.9	61	41.5
G/G	G/G	Wild type	Deleted	8	15.4	14	9.5
G/G	G/G	Polymorphic	Present	2	3.8	3	2.0
G/G	G/G	Polymorphic	Deleted	1	1.9	1	0.7
G/G	G/C+C/C	Wild type	Present	4	7.7	19	12.9
G/G	G/C+C/C	Wild type	Deleted	4	7.7	1	0.7
G/A+A/A	G/G	Wild type	Present	0	0.0	0	0.0
G/A+A/A	G/G	Wild type	Deleted	0	0.0	0	0.0
G/A+A/A	G/C+C/C	Wild type	Present	2	3.8	0	0.0
G/A+A/A	G/C+C/C	Wild type	Deleted	0	0.0	1	0.7
G/A+A/A	G/G	Polymorphic	Present	0	0.0	0	0.0
G/A+A/A	G/G	Polymorphic	Deleted	0	0.0	0	0.0
G/G	G/C+C/C	Polymorphic	Present	0	0.0	0	0.0
G/G	G/C+C/C	Polymorphic	Deleted	0	0.0	0	0.0
G/A+A/A	G/C+C/C	Polymorphic	Present	15	28.8	34	23.1
G/A+A/A	G/C+C/C	Polymorphic	Deleted	2	3.8	13	8.8

Monte Carlo $\chi^2 = 20.66$, $p = 0.008$.
G/G, wild type.

The main finding of this study was the presence of a significantly lower frequency of certain combined genotypes in centenarians, such as *GSTT1* deletion/*p53* 16 bp duplication, and *GSTT1* deletion/*p53* Msp1 polymorphism. A possible explanation for these findings is that loss of function in *GSTT1* allows the accumulation of a large amount of activated carcinogens, which are known to induce several types of DNA damage such as DNA adducts. In addition, polymorphisms in *p53* are known to affect DNA repair and genome stability (Wani *et al.* 2000). Subjects with both *GSTT1* deletion and *p53* polymorphisms could therefore have greater exposure to carcinogens and reduced DNA repair ability, and thus are at increased risk for cancer. This mechanism could also explain why the pattern of deletion of the two phase II genes *GSTM1* and *GSTT1* together with *p53* polymorphisms was very rare, or even absent, in our population of centenarians. The deletion of two glutathione transferases would amplify the carcinogenic effect described above. Case–control studies have shown an increased risk of lung cancer associated with the *GSTs1* and *p53* double variants (Liu *et al.* 2001, Miller *et al.* 2002). It is possible that the effect of the combined genotypes is greater than the sum of the independent effects, even though the genes are not on the same biological pathways; multiple variants may interact in a unique way and produce a biological effect for which individual variants may have poor predictive power (Kinzler and Vogelstein 1997).

No data was available on the past environmental exposure in this population; however, it is likely that environmental conditions were very different at the beginning of the century, when this population was young and active, than nowadays. Our study included a large number of female centenarians, whose reproductive life included more children than younger females, and this could be a contributing factor to longevity.

Another possible explanation for our findings is that both metabolic genes and *p53* act on pathways related to cell ageing and death. It is known that *p53* acts on apoptosis and cell senescence. It is possible that the accumulation of oxidative processes, such as those found in the presence of deletion of the *GSTs* genes, could increase the mechanisms of cell senescence and death associated with *p53* polymorphisms. In this case, certain composite genetic patterns would represent a generic mechanism of protection against ageing, not just against the development of chronic diseases. Since cancer is not the only disease responsible for a short survival, it is possible that the genes included in our study could also be relevant to other pathological processes.

One aspect that needs to be underlined is that our study indicates that combinations of polymorphisms in several different classes of genes need to be studied when looking at the processes of carcinogenesis or ageing. The pathways we explored by looking at some candidate genes are probably very complex and involve several other gene families. Large samples of healthy centenarians are needed in order to study multiple pathways and several gene polymorphisms at once. In conclusion, it is likely that longevity is related to a very complex genetic trait as well as to certain environmental exposures.

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