Characterization of Novel and Uncharacterized p53 SNPs in the Chinese Population – Intron 2 SNP Co-Segregates with the Common Codon 72 Polymorphism

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Abstract

Multiple single nucleotide polymorphisms (SNPs) have been identified in the tumor suppressor gene p53, though the relevance of many of them is unclear. Some of them are also differentially distributed in various ethnic populations, suggesting selective functionality. We have therefore sequenced all exons and flanking regions of p53 from the Singaporean Chinese population and report here the characterization of some novel and uncharacterized SNPs – four in intron 1 (nucleotide positions 8759/10361/10506/11130), three in intron 3 (11968/11969/11974) and two in the 3’UTR (19168/19514). Allelic frequencies were determined for all these and some known SNPs, and were compared in a limited scale to leukemia and lung cancer patient samples. Intron 2 (11827) and 7 (14181/14201) SNPs were found to have a high minor allele frequency of between 26–47%, in contrast to the lower frequencies found in the US population, but similar in trend to the codon 72 polymorphism (SNP12139) that shows a distribution pattern correlative with latitude. Several of the SNPs were linked, such as those in introns 1, 3, and 7. Most interestingly, we noticed the co-segregation of the intron 2 and the codon 72 SNPs, the latter which has been shown to be expressed in an allele-specific manner, suggesting possible regulatory cross-talk. Association analysis indicated that the T/G alleles in both the co-segregating intron 7 SNPs and a 4tagSNP haplotype was strongly associated increased susceptibility to lung cancer in non-smoker females [OR: 1.97 (1.32, 3.394)]. These data together demonstrate high SNP diversity in p53 gene between different populations, highlighting ethnicity-based differences, and their association with cancer risk.

Introduction

The tumor-suppressor gene p53 is highly mutated up to 50% in all human cancer types [1], making it the most genetically targeted gene involved in carcinogenesis. Mutations often lead to loss of the tumor-suppressive functions of p53 - especially the transcription-dependent functions, highlighted by the enormous hot-spot mutations in the DNA-binding domain - thereby leading to uncontrolled cellular growth [1–3]. Besides mutations, p53 is thought also to be functionally inactivated through deregulated upstream and downstream regulators [1–3]. A case in point is the overexpression of its negative regulator MDM2, which leads to the rapid degradation of p53 [4–6]. In addition, p53 can also be functionally incapacitated by altered activation mechanisms involving post-translational modifications [7,8]. p53 therefore is not mutated in these cases but is unable to perform its functions optimally due to lack of activation, as has also been noted in the case of neuroblastomas where it is often sequestered in the cytoplasm [9].

Being the most important tumor suppressor gene, the association of p53 status with cancer risk has always been a captivating area of work. As mutations do not lead to elevated non-familial cancer risk in population-based studies (except in the Li-Fraumeni syndrome families with germ-line p53 mutations), the role of single nucleotide polymorphisms (SNPs) in p53 and their association with cancer risk has been studied extensively. It is noteworthy that there are about 90 SNPs reported in the p53 gene, of which only six are in coding exons [10]. The relevance of the majority of the intronic SNPs is at present unclear. However, a few of the intronic SNPs, in particular, those in intron 3 (SNP11951) and intron 6 (SNP13494 and 13964) has been associated with cancer predisposition [11–14]. Nonetheless, the functional relevance of the different intronic SNPs in affecting cancer risk is not well understood. Moreover, most of the other intronic SNPs have not been well characterized, or little information including allelic frequency in various populations is available to understand their significance. Of the exonic SNPs, only two, in exon 4, are non-synonymous SNPs leading to altered amino-acids (SNP12063 encoding for codon 47 and SNP12139 coding for codon 72) [15]. Both these polymorphisms have been suggested to affect p53 function to varying extents, though the relevance of the codon 72 polymorphism has been extensively studied over the last 2 decades.
The codon 72 polymorphism has been controversially associated with cancer risk in many cases. Essentially, both the G (giving rise to an arginine amino-acid) or C (giving rise to a proline amino-acid) alleles have been found to be associated with various types of cancer risks, though such associations have not been consistently noted in different populations [17–26]. Interestingly, there is a clear trend in the distribution of these polymorphic alleles, and a correlation exists between the presence of the G allele and distance away from the Equator [27]. Therefore, populations such as the Africans who live closer to the Equator tend to have a larger proportion of C-allele carriers compared to the Northern Europeans who are predominantly the G-allele carriers [27]. Thus, ethnicity has been suggested to be a critical factor in determining the effects of these different alleles on cancer predisposition [18,20,23], underlining the need for understanding the allelic distribution in various populations.

Functionally, the different codon 72 polymorphic variants have been shown to affect p53 function differentially. The arginine-variant was found to have a higher ability to induce mitochondrial-mediated apoptosis [28]. In contrast, the proline-variant was found to have a better capacity to induce DNA-repair and cell-cycle arrest, by virtue of differential activation of p53-target genes [29,30]. Moreover, these variants have differing affinities to protein partners such as iASPP and MDM2 [28,31], thereby suggesting that the structural changes may influence the function of these p53 forms.

We have previously reported that there is an allele-specific expression of the codon72-polymorphic variant p53 forms, both in heterozygous normal and cancer cohorts [17]. In normal healthy individuals, the proline-variant was preferentially expressed in contrast to the preferential expression of the arginine-variant in the breast cancer heterozygote cases [17]. How this allele-specific expression is regulated is unclear. Thus, in an attempt to investigate if there are other regulatory regions that may have relevance in determining the preferential expression of the codon 72 polymorphic variants, we also noted association of the intron 7 SNPs with cancer risk, both alone or in the haplotype analysis in a limited scale study of female non-smoker lung-cancer samples. Detailed results are presented.

Results

Identification of novel SNPs in p53 and their allelic frequencies

We sequenced the regions covering all the exons and their flanking regions of the p53 gene of about 11 kb from genomic DNA of healthy volunteers, from intron 1 (from nucleotide position 8634) to the 3′UTR region (19715), and identified several intronic SNPs that have not been previously reported or have been reported but not characterized. These included four in intron 1 (at positions 8759, 10361, 10506 and 11130), three in intron 3 (11968, 11969 and 11974) and two in the 3′UTR (19168 and 19514). Comparison with HapMap data indicated that of these, one SNP in intron 1 (10506) and all three in intron 3 (11968, 11969 and 11974) have not been previously reported. Table 1 shows the SNPs in p53 that we identified, together with some of the others that are already known and is the subject of this study. The SNP alleles specifically characterized in this study are as follows: in intron 1 - 8759T/C, 10361G/A, 10506T/C and 11130A/G; intron 3 – 11968G/A, 11969G/C and 11974G/A; 3′UTR – 19168G/A and 19514G/A.

Table 1. List of SNPs analyzed among Chinese healthy controls.

| SNP (Intron/exon position) | Minor allele | Major allele | Minor allele frequency (95% CI) | Minor allele frequency from HapMap (US population) | Minor allele frequency from HapMap (Chinese population) |
|---------------------------|--------------|--------------|---------------------------------|-----------------------------------------------|---------------------------------------------------|
| in.1…8759                | C            | T            | 0.292 (0.214, 0.385)            | 0.19                                          | Not reported                                      |
| in.1…10361               | A            | G            | 0.311 (0.231, 0.404)            | 0.29                                          | Not reported                                      |
| in.1…10506               | C            | T            | 0.274 (0.198, 0.366)            | Not reported                                  | Not reported                                      |
| in.1…11130               | A            | G            | 0.292 (0.214, 0.385)            | 0.17                                          | Not reported                                      |
| in.2…11827               | C            | G            | 0.470 (0.355, 0.589)            | 0.25                                          | Not reported                                      |
| in.3…11968               | A            | G            | 0.009 (0.002, 0.051)            | Not reported                                  | Not reported                                      |
| in.3…11969               | C            | G            | 0.009 (0.002, 0.051)            | Not reported                                  | Not reported                                      |
| in.3…11974               | A            | G            | 0.009 (0.002, 0.051)            | Not reported                                  | Not reported                                      |
| in.3…11992               | A            | C            | 0.029 (0.010, 0.082)            | 0.05                                          | Not reported                                      |
| Ex.4…12139               | C            | G            | 0.470 (0.355, 0.589)            | 0.35                                          | 0.50                                              |
| in.6…13494               | A            | G            | 0.028 (0.01, 0.079)             | 0.06                                          | 0.034                                             |
| in.7…14181               | T            | C            | 0.264 (0.189, 0.355)            | 0.16                                          | Not reported                                      |
| in.7…14201               | G            | T            | 0.264 (0.189, 0.355)            | 0.083                                         | 0.39                                              |
| 3′UTR.19168              | A            | G            | 0.117 (0.067, 0.197)            | 0.10                                          | 0.044                                             |
| 3′UTR.19514              | A            | G            | 0.052 (0.022, 0.116)            | 0.04                                          | Not reported                                      |

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Novel p53 SNPs in the Chinese Population
The allelic frequencies of these novel and some of the other known SNPs were determined using samples from healthy Chinese population [17] (Table 1). All SNPs in controls were in Hardy-Weinberg Equilibrium. Minor allele frequencies (MAF) of each SNP, together with 95% CI are listed in Table 1. Among these 15 SNPs analyzed (including the novel ones), three SNPs at intron 3 (11968, 11969, 11974) had MAF of less than 1%. Of the remaining 12 SNPs, the MAF ranges from 2.8% (SNP 13494) to 47.0% (11827 and 12139 [codon 72]). Of note, several SNPs in introns 1, 2 and 7 had MAF between 26–46%, in contrast to lower frequencies reported in the US population [10], suggesting that these SNPs may have important functional relevance in p53 biology in a population-dependent manner.

Co-segregating SNPs

Of the newly characterized SNPs, several of them co-segregated. The pattern of pairwise linkage disequilibrium (LD) between the various SNPs among controls are shown in Figure 1. For example, the intron 1 SNPs 8759T/C co-segregated with SNP 10361G/A (1602 nucleotides apart) and SNP 10506T/C (1747 nucleotides apart) (Figure 1). The SNPs at position 8759, 10361 and 10506 in intron 1 were in strong LD to each other with $r^2$ greater than 0.8. Similarly, the three SNPs in intron 3 including SNP11968, 11969 and 11974 were in complete LD to each other (all $r^2 = 1$) (Figure 1).

We also identified a linkage between previously noted SNPs, such as that in intron 7 [32]. The two SNPs in intron 7 (14181 and 14201) were also in complete LD ($r^2 = 1$). Moreover, we also found that the SNP in intron 2 (11827) co-segregated with the common codon 72 polymorphic SNP12139 in exon 4 (Figure 1). A noteworthy point is that the intervening coding regions, which contain the SNPs in intron 3, were not linked with these alleles. Moreover, the intron 7 SNPs have some level of LD with the intron 1 and 2 SNPs ($r^2>0.6$), though the intervening SNP13494 site in intron 6 was not linked with them, highlighting gene rearrangements in the p53 gene. Thus, the data analyses have uncovered previously unnoticed LD associations, especially between the intron 2 SNP11827 and the codon 72 SNP12139, the latter which has been associated with cancer predisposition [19,21,22,25,26].

SNPs and association with cancer susceptibility

In order to determine if the newly identified SNPs have any association with cancer susceptibility, we analyzed the distribution of some of the alleles of the SNPs in healthy normal donors, and in a pilot study with a small group of leukemia patients or in a group of non-smoker female lung cancer patients, which have been used in our previous p53 association studies. The leukemia group analyzed consisted of up to 44 Chinese patients whose DNA were previously used for analysis of the effect of p53 codon 72 polymorphism (in exon 4), which revealed no significant association of this polymorphism on leukemia susceptibility [33]. The lung cancer cohort consisted of up to 79 patient samples, also utilized in a previous study analyzing the effects of p53 codon 72...
Table 2. Risk estimates (OR* and 95% CI*) for leukemia for individual SNP.

| Intron/Exon...SNP | Model | Controls | Cases | OR (95% CI) | GlobalP | P trend |
|-------------------|-------|----------|-------|-------------|---------|---------|
|                   |       | n | % | n | % |         |         |
| In.3...11968      | Genotype | 0 | 0.0 | 0 | 0.0 | 1.000 | 0.349 |
|                   | A      | 1 | 1.9 | 0 | 0.0 | 0.376 (0.015, 9.465) |         |
|                   | G      | 52 | 98.1 | 44 | 100.0 |         |         |
| Allele            | A      | 1 | 0.9 | 0 | 0.0 | 0.380 (0.015, 9.447) |         |
|                   | G      | 105 | 99.1 | 88 | 100.0 |         |         |
| In.3...11969      | Genotype | 0 | 0.0 | 0 | 0.0 | 1.000 | 0.349 |
|                   | C      | 1 | 1.9 | 0 | 0.0 | 0.376 (0.015, 9.465) |         |
|                   | G      | 52 | 98.1 | 44 | 100.0 |         |         |
| Allele            | C      | 1 | 0.9 | 0 | 0.0 | 0.380 (0.015, 9.447) |         |
|                   | G      | 105 | 99.1 | 88 | 100.0 |         |         |
| In.3...11974      | Genotype | 0 | 0.0 | 0 | 0.0 | 1.000 | 0.349 |
|                   | A      | 1 | 1.9 | 0 | 0.0 | 0.376 (0.015, 9.465) |         |
|                   | G      | 52 | 98.1 | 44 | 100.0 |         |         |
| Allele            | A      | 1 | 0.9 | 0 | 0.0 | 0.380 (0.015, 9.447) |         |
|                   | G      | 105 | 99.1 | 88 | 100.0 |         |         |
| In.3...11992      | Genotype | 0 | 0.0 | 0 | 0.0 | 1.000 | 0.750 |
|                   | AC     | 3 | 5.8 | 2 | 4.5 | 0.742 (0.119, 4.651) |         |
|                   | CC     | 49 | 94.2 | 42 | 95.5 |         |         |
| Allele            | A      | 3 | 2.9 | 2 | 2.3 | 0.748 (0.122, 4.579) |         |
|                   | C      | 101 | 97.1 | 86 | 97.7 |         |         |
| In.6...13494      | Genotype | 0 | 0.0 | 0 | 0.0 | 0.675 | 0.570 |
|                   | AC     | 3 | 5.7 | 3 | 8.8 | 1.613 (0.306, 8.498) |         |
|                   | CC     | 50 | 94.3 | 31 | 91.2 |         |         |
| Allele            | A      | 28 | 26.4 | 15 | 32.6 | 1.348 (0.635, 2.861) |         |
|                   | T      | 78 | 73.6 | 31 | 67.4 |         |         |
| In.7...14181      | Genotype | 0 | 0.0 | 0 | 0.0 | 0.641 | 0.435 |
|                   | TT     | 4 | 7.5 | 2 | 8.7 | 1.450 (0.230, 9.160) |         |
|                   | TC     | 20 | 37.7 | 11 | 47.8 | 1.595 (0.570, 4.461) |         |
|                   | CC     | 29 | 54.7 | 10 | 43.5 |         |         |
| Allele            | T      | 28 | 26.4 | 15 | 32.6 |         |         |
|                   | C      | 78 | 73.6 | 31 | 67.4 |         |         |
| In.7...14201      | Genotype | 0 | 0.0 | 0 | 0.0 | 0.641 | 0.435 |
|                   | GG     | 4 | 7.5 | 2 | 8.7 | 1.450 (0.230, 9.160) |         |
|                   | GT     | 20 | 37.7 | 11 | 47.8 | 1.595 (0.570, 4.461) |         |
|                   | TT     | 29 | 54.7 | 10 | 43.5 |         |         |

*OR* and 95% CI calculated for each SNP genotype compared to the reference genotype.
polymorphism and MDM SNP309, which found no significant association of the codon 72 SNP with lung cancer susceptibility in female [34].

Association tests of each SNP showed that none of the analyzed SNPs were associated with leukemia risk (Table 2), while 2 of 6 SNPs (14181 and 14201) investigated were found to have significant association with increased lung cancer risk (Table 3).

Based on the LD pattern among the 6 SNPs investigated in lung cancers with MAF at least 1%, 4 tagSNPs (SNPs 11827, 11992, 13494 and 14201) were selected for further haplotype analyses. The two most common haplotypes were GCCG (lung cancer: 58.6%, control: 64.7%) and CCGG (case: 23.8%, control: 26.1%) (Table 4). Single omnibus test detected a significant association of these haplotype with lung cancer (p = 0.036). Further haplotype-specific test showed that haplotype GCCG increased lung cancer risk significantly (p = 0.015) when compared to the other haplotypes (Table 4). Similar analysis of leukemia patients did not reveal any evidence of association with leukemia (data not shown).

These data together indicate that intron 7 SNPs 14181 and 14201 show an association with lung cancer risk in the Chinese female non-smoker population. Moreover, the haplotype analyses also indicate a correlation between the 4 tagSNP and increased lung cancer probability, highlighting that intronic SNPs may play a role in cancer susceptibility.

Intron 7 SNPs and overall survival

We therefore performed Kaplan-Meier analysis to determine if the different genotypes of the intron 7 SNPs would affect overall survival (OS). Median OS were found to be 1.69, 0.62 and 0.93 years for lung cancer patients with genotype C/C, C/T and T/T at SNP 14181, respectively (Figure 2A), while it was 0.93, 0.70 and 1.69 years for patients with genotypes G/G, G/T and T/T at SNP14201, respectively (Figure 2B). Log-rank tests did not detect any significant difference in OS among different genotypes at SNPs14181 (p = 0.112) and 14201 (p = 0.108). These data therefore indicate that although the intron 7 SNPs are associated with risk of lung cancer, there are no notable associations with OS in this limited study.

Discussion

This work was undertaken to perform a detailed analysis of SNPs in the p53 gene in the Chinese population, with the aim of uncovering new SNPs as well as to study previously noted SNPs in p53 that have not been characterized in the Chinese cohorts [10]. Although many SNPs have been reported in the HapMap project, most contain only data from the US population (which comprises of 24 African Americans, 24 Asian Americans, 24 European Americans, 12 Hispanic American, 6 Native Americans) [10]. Our analysis confirmed the presence of SNPs noted in the HapMap project, and also uncovered several novel SNPs in introns 1 and 3. In addition, we focused our attention on other related and interesting SNPs, such as those in introns 2, 7 and 11, and have identified complete LD between the known codon 72 polymorphism (SNP12139) in exon 4 and the SNP11827 in intron 2, suggesting that they may be functionally related. Furthermore, we have noticed a strong correlation between the two linked SNPs in intron 7 with lung cancer predisposition.

When the allelic frequencies from our study was compared to the HapMap data on Chinese subjects, there was a general correlation, as evidenced by the exon 4 SNP12139, intron 6 SNP13494 and intron 7 SNP14201 (Table 1), validating this analysis. Based on this, further comparison of reported frequencies in the US population with the Chinese population revealed strong differences in two sets of SNPs; the intron 2 (11827)/exon 4 (12139) and the intron 7 SNPs (14181/14201). Essentially, there is an increase of the minor allele in the Chinese population compared to the US population in the above cases, similar to the originally described trend of an association of the exon 4 (codon 72) SNP with populations at different latitudes [27]. This therefore highlights that ethnicity is a critical factor in the distribution of allelic frequencies, and may be functionally relevant in determining p53 function.

The most striking observation was the identification of the linkage of the intron 2 SNP (11827) with the common codon 72 SNP in exon 4 (12139), which were in complete LD with each other. It was interesting that this was not noticed earlier, given the fact that the codon 72 SNP has been extensively studied with respect to cancer susceptibility. We have previously reported that the arginine or the proline variants of the exon 4 polymorphism, which arise due to the G or C nucleotides respectively, can be differentially expressed in healthy and cancer cases [17]. The regulation of the allele-specific differential expression is at present unclear. Thus, the recognition of the linkage between this SNP and that in intron 2, especially with the C or G nucleotides co-segregating in both cases, may provide us with some clues to the regulation of the codon 72 SNP-specific expression, provided that the intron 2 SNP has transcription...
Table 3. Risk estimates (OR and 95% CI) for lung cancer for individual SNP.

| SNP     | Model | Controls | Cases | OR (95% CI)       | Global P | P trend |
|---------|-------|----------|-------|-------------------|----------|---------|
|         |       | n %      | n %   |                   |          |         |
| In.2...11827 Genotype |       |          |       |                   |          |         |
| CC      | 15    | 28.3     | 16    | 22.5              | 0.782    | 0.621   |
| CG      | 27    | 50.9     | 40    | 56.3              | 1.086    |         |
| GG      | 11    | 20.7     | 15    | 21.1              |          |         |
| Allele  | C     | 57       | 53.8  | 72                | 1.131    | 0.683   |
|         | G     | 49       | 46.2  | 70                | 1.086    |         |
| In.3...11992 Genotype |       |          |       |                   |          |         |
| AA      | 0     | 0.0      | 1     | 4.183 (0.166, 105.695) | 0.119    | 0.070   |
| AC      | 3     | 5.8      | 6     | 2.800 (0.655, 11.963) |          |         |
| CC      | 49    | 94.2     | 35    |                   | 0.066    |         |
| Allele  | A     | 3        | 2.9   | 8                 | 3.544    | 0.910   |
|         | C     | 101      | 97.1  | 76                |          |         |
| Ex.4...12139 Genotype |       |          |       |                   |          |         |
| CC      | 15    | 28.3     | 16    | 22.5              | 0.782    | 0.621   |
| CG      | 27    | 50.9     | 40    | 56.3              | 1.086    |         |
| GG      | 11    | 20.7     | 15    | 21.1              |          |         |
| Allele  | C     | 57       | 53.8  | 72                | 1.131    | 0.683   |
|         | G     | 49       | 46.2  | 70                | 1.086    |         |
| In.6...13494 Genotype |       |          |       |                   | 0.312    | 0.185   |
| AA      | 0     | 0.0      | 0     |                   |          |         |
| AG      | 3     | 5.7      | 1     | 0.238 (0.024, 2.356) | 0.316    |         |
| GG      | 50    | 94.3     | 70    |                   |          |         |
| Allele  | A     | 3        | 2.8   | 1                 | 0.243    | 0.0250  |
|         | G     | 103      | 97.2  | 141               |          |         |
| In.7...14181 Genotype |       |          |       |                   | 0.018    | 0.011   |
| TT      | 4     | 7.5      | 8     | 2.900 (0.768, 10.949) |          |         |
| TC      | 20    | 37.7     | 41    |                   | 2.973    | 1.361   |
| CC      | 29    | 54.7     | 20    |                   |          | 6.492   |
| Allele  | T     | 28       | 26.4  | 57                | 1.960    | 1.132   |
|         | C     | 78       | 73.6  | 81                |          | 3.394   |
| In.7...14201 Genotype |       |          |       |                   | 0.014    | 0.010   |
| GG      | 4     | 7.5      | 8     | 2.900 (0.768, 10.949) |          |         |
| GT      | 20    | 37.7     | 42    |                   | 3.045    | 1.396   |
| TT      | 29    | 54.7     | 20    |                   |          | 6.641   |
| Allele  | G     | 28       | 26.4  | 58                | 1.970    | 1.140   |
|         | T     | 78       | 73.6  | 82                |          | 3.406   |

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regulatory functions. This hypothesis is currently being tested. Moreover, the relevance of the intron 2 SNP has not been well characterized yet, especially with respect to cancer susceptibility, but given the recognition of complete LD with the exon 4 SNP, future work should shed light on its functional relevance. It is also noteworthy that the intervening SNPs in intron 3 are totally not linked to the intron 2 and codon 72 SNPs, suggesting rearrangements in the human $p53$ gene have not affected this SNP pair, tempting us to speculate that this co-segregation may be important in regulating $p53$ functions.
Table 4. Four marker haplotype frequencies and association with lung cancer.

| No | Haplotype | In.2... 11827 | In.3... 11992 | In.6... 13494 | In.7... 14201 | Frequency | $\chi^2$ | P value |
|----|-----------|----------------|----------------|----------------|----------------|-----------|--------|--------|
| 1  | G C G T   | 0.586          | 0.647          | 0.696          | 0.404          | 0.675     | 0.404  |
| 2  | C C G G   | 0.238          | 0.261          | 0.124          | 0.725          | 0.32     | 0.725  |
| 3  | G C G G   | 0.063          | 0.003          | 5.884          | 0.015          | 0.000     | 0.015  |
| 4  | C C A T   | 0.095          | 0.028          | 3.650          | 0.056          | 0.019     | 0.056  |
| 5  | C A G T   | 0.019          | 0.032          | 0.317          | 0.573          | 0.00     | 0.573  |
| 6  | C C G T   | 0.019          | 0.032          | 0.317          | 0.573          | 0.000     | 0.573  |

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Figure 2. Kaplan-Meier curves of overall survival of lung cancer patients with different genotypes at intron 7, SNP14181 (A) or SNP14201 (B).
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We also attempted to compare the various SNPs with cancer susceptibility in a limited scale using samples from our previous studies on leukemia and female non-smoker lung cancer patients [32,33]. Many of the SNPs characterized were not found to have any significant association with cancer risk. However, we noticed that the variant alleles of the intron 7 SNPs were associated with increased risk in the lung-cancer cohorts. Although these two SNPs were noted earlier, it was not recognized that they were completely linked [34]. Of these two SNPs, the SNP 14181 result may also regulate splicing of p53, resulting in the various splice variants that are emerging as important regulators of cell survival [37]. Hence, future work should shed light on the functional relevance of these intronic SNPs in regulating cancer risk.

Materials and Methods

Samples

Genomic DNA were prepared from peripheral blood as described by standard procedures using the Qiagen kit, from a total of 53 healthy individuals [17], and up to 44 leukemia patients [32] and up to 79 non-smoker lung-cancer patients [33], as have been described in our previous studies. Study was conducted informed written consent from participants with the approval of the National Cancer Centre Singapore and the Department of Hematology, Singapore General Hospital ethics committees and the Institutional Review Board of the National University of Singapore. The genomic DNA was used for genotyping and the data on overall survival was obtained from the hospital records with ethics approval.

Details of the leukemia and lung cancer group have been described in our previous studies aimed at determining the association between codon 72 polymorphism (exon 4 SNP) and cancer susceptibility in these populations [32,33]. Essentially, all are Chinese Singaporeans, diagnosed at any one of three major hospitals in the country over the study period, and the demographic, smoking and other relevant information was obtained by in-person interview with a trained nurse, as described [32,33].

Genotyping and Sequencing

Genomic DNA was sequenced from nucleotide position 8634 at the 5' end of p53 to nucleotide 19715 at the 3' end of p53, covering...
Statistical Analysis
The observed genotype frequencies of each SNP in the controls were tested for Hardy-Weinberg equilibrium (HWE) using Fisher’s exact test. The frequency of minor allele at each SNP in HWE is reported together with 95% Confidence Interval (CI). P value on the basis of the 3 categorical genotypes, or allele variable, and a p trend value on the basis of the 3 level ordinal genotype variable (0: wildtype homozygotes, 1: heterozygotes, 2: mutant homozygotes) in the logistic regression model were calculated. SNPs with minor allele frequency (MAF) of at least 1% among control sample were used to select tagSNPs based on pairwise r² linkage disequilibrium (LD) map [38] using Tagger in Haploview version 4.0 (http://www.broad.mit.edu/mpg/haploview) [39], at a relatively stringent r² threshold of 0.8. Haplotype analysis of these tagSNPs in this case-control study was carried out using PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) [40]. For each haplotype with frequency at least 1% either in case or control, a single omnibus test was performed to test the effects of all these haplotypes jointly, and haplotype-specific test with 1 degree-of-freedom was also carried out for each haplotype using PLINK by comparing this specific haplotype with all others.

Kaplan-Meier estimated overall survival (OS) was carried out and log-rank test was used to compare the equivalence of OS among different genotypes at the two SNPs of intron 7. Kaplan-Meier estimated median OS were reported for each genotype at these two SNPs. All analyses were done using R version 2.7.1 (http://www.R-project.org).

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Author Contributions
Conceived and designed the experiments: BHP KS. Performed the experiments: BHP HWG. Analyzed the data: BHP HL KS. Contributed reagents/materials/analysis tools: YCL. Wrote the paper: KS.

References
1. Petitjan A, Achatz MJ, Borsgers-Dale AL, Haimau P, Olivier M (2002) TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Review. Oncogene 26(15): 2157–2165.
2. Vouten KH, Lu X (2002) Live or let die: the cell’s response to p53. Nat Rev Cancer 2: 594–604.
3. Brosh R, Rotter V (2009) When mutants gain new powers: news from the iASPP pathway. Oncogene 28: 6135–6140.
4. Haupt Y, Maya R, Kazar Z, Oren M (1997) Mdm2 promotes the rapid degradation of p53. Nature 387: 296–299.
5. Watanabe T, Ichikawa A, Saito H, Hotta T (1996) Overexpression of the MDM2 oncogene in leukemia and lymphoma. Leuk Lymphoma 21: 391–397.
6. Kupper M, Joss S, von Bonin F, Dass H, Piurencschuh M, et al. (2001) MDM2 gene amplification and lack of p53 point mutations in Hodgkin and Reed-Sternberg cells: results from single-cell polymerase chain reaction and molecular cytogenetic studies. Br J Haematol 112: 768–775.
7. Tan J, Zhuang L, Leong HS, Iyer NG, Liu ET, et al. (2005) Pharmacologic modulation of glycogen synthase kinase-3beta promotes p53-dependent apoptosis through a direct Iex-mediated mitochondrial pathway in colorectal cancer cells. Cancer Res 65(19): 9012–9020.
8. Lavin MF, Khamma KK (1999) ATM: the protein encoded by the gene mutated in the radiosensitive syndrome ataxia-telangiectasia. Int J Radiat Biol 75(10): 1201–1214.
9. Moll UM, LaQuaglia M, Be´nard J, Riou G (1995) Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumours. Proc Natl Acad Sci USA 92: 4407–4411.
10. The International HapMap Consortium (2003) The International HapMap project. Nature 426: 789–796.
11. Wang-Gehrike S, Weikel W, Risch H, Vesprini D, Abrahamson J, et al. (1999) Intron variants of the p53 gene are associated with increased risk for ovarian cancer but not in carriers of BRCA1 or BRCA2 germ-line mutations. Br J Cancer 81(1): 179–183.
12. Gemignani F, Murro V, Landi S, MoUllan N, Chabrier A, et al. (2004) A TP53 polymorphism is associated with increased risk of colorectal cancer and with reduced levels of TP53 mRNA. Germ line polymorphisms of the tumor suppressor gene p53 and lung cancer. Oncogene 23(10): 1954–1963.
13. Rippa F, Zanini F, Kozhit A, Shubja J, Salagovic J (2001) Germ line polymorphisms of the tumor suppressor gene p53 and lung cancer. Lung Cancer 31(2-3): 157–162.
14. Lehman TA, Haffty BG, Carbone CJ, Bishop LR, Gumbs AA, et al. (2000) Elevated frequency and functional activity of a specific germ-line p53 intron mutation in familial breast cancer. Cancer Res 60(4): 1062–1069.
15. Piekst EC, Humbley O, Murphy ME (2006) Polymorphisms in the p53 pathway. Oncogene 25: 1602–1611.
16. Whichelby G, Phay CJ, Hallmamn PD, Holleimn M (2000) TP53 polymorphisms: cancer implications. Int J Cancer 92(2): 95–107.
17. Siddique MM, Balbra¨n C, Fizzer-Malzisewska L, Aggarwal A, Tan A, et al. (2005) Evidence for selective expression of the p53 codon 72 polymorphism: implications in cancer development. Cancer Epidemiol Biomarkers Prev 14: 2243–2252.
18. Lum SS, Chua HW, Li H, Li WF, Rao N, et al. (2008) MDM2 SNP309G allele increases risk but the T allele is associated with earlier onset age of sporadic breast cancers in the Chinese population. Carcinogenesis 29(4): 754–761.
19. Toyama T, Zhang Z, Nishio M, Hamaguchi M, Kondo N, et al. (2007) Association of TP53 codon 72 polymorphism and the outcome of adjuvant therapy in breast cancer patients. Breast Cancer Res 9: R84.
20. Khadang B, Fahansi MJ, Tali A, Delahagn A, Ghaderi A (2007) Polymorphism of TP53 codon 72 showed no association with breast cancer in Iranian women. Cancer Genet Cytogenet 173: 38–42.
21. Papadakis EN, Dokianakis DN, Spandidos DA (2000) P53 codon 72 polymorphism as a risk factor in the development of breast cancer. Mol Cell Biol Res Commun 3: 389–392.
22. Damra AP, Frazson AP, Damra DC, Roehe A, Hermes V, et al. (2006) Evidence for an association of TP53 codon 72 polymorphism with breast cancer risk. Cancer Detect Prev 30: 328–329.
23. Mahboubi I, Baccouche S, El-Abed R, Mokdad-Gargouri R, Moshal A, et al. (2003) No evidence of correlation between p53 codon 72 polymorphism and risk of bladder or breast carcinoma in Tunisian patients. Ann N Y Acad Sci 1010: 764–770.
24. Buyu N, Togly H, Dala Y, Nai P (2003) P53 codon 72 polymorphism in breast cancer. Oncol Rep 10: 711–714.
25. Wu X, Zhao H, Amos CI, Shete S, Makan N, et al. (2002) P53 Genotypes and haplotypes associated with lung cancer susceptibility and ethnicity. J Natl Cancer Inst 94: 681–690.
26. Wang YC, Chen CY, Chen SK, Chang YY, Lin P (1999) p53 codon 72 polymorphism in Taiwanese lung cancer patients: association with lung cancer susceptibility and prognosis. Clin Cancer Res 5: 129–134.
27. Beckman G, Birgander R, Sjolander A, Saha N, Holmberg PA, et al. (1994) Is P53 polymorphism maintained by natural selection? Hum. Hered 44: 266–270.
28. Dumont P, Lee JL, Della Pietra AC, 3rd, George DL, Murphy M (2003) The codon 72 polymorphism variants of p53 have markedly different apoptotic potential. Nat Genet 35: 357–363.
29. Siddique M, Sabapathy K (2006) Trp53-dependent DNA-repair is affected by the codon 72 polymorphism. Oncogene 25: 3490–3500.
30. Fim D, Banks I (2004) P53 polymorphism variants at codon 72 exert different effects on cell cycle progression. Int J Cancer 108: 196–199.
31. Bergamaschi D, Samuels Y, Sullivan A, Zveleblj H, Brysens H, et al. (2006) iASPP preferentially binds p33 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53. Nat Genet;10: 1133–1141.
32. Pim D, Banks L (2004) P53 polymorphic variants at codon 72 exert different effects on cell cycle progression. Int J Cancer 108: 196–199.
33. Chua HW, Ng D, ChooS, Lum SS, Li H, et al. (2010) Effect of MDM2 SNP309 and p53-codon 72 polymorphisms on lung cancer risk and survival among non-smoking Chinese women in Singapore. BMC Cancer 10: 88.
34. Prosser J, Condie A (1991) Biallelic Apal polymorphism of the human p53 gene (TP53). Nucleic Acids Res 19: 4799.
35. Gu QH, Chen Q, Ho CP, Li YQ, Yang HZ (2007) Study on the relationship between the polymorphism of p53 gene intron 7 and non-small cell lung cancer (NSCLC) and p53 mutation in NSCLC tissues. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 24(3): 319–321.
36. Li YQ, Li YL, Gu H, Ye AH, Wu TS (2005) p53 gene intron 7 polymorphism and its association with oral neoplasms. Zhonghua Kou Qiang Yi Xue Za Zhi 40(5): 386–389.
37. Bourdon JC (2007) p53 and its isoforms in cancer. Br J Cancer 97(3): 277–282.
38. Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, et al. (2004) Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. Am J Hum Genet 74: 106–120.
39. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21(2): 263–265.
40. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, et al. (2007) PLINK: a toolset for whole-genome association and population-based linkage analysis. Am J Hum Genet 81: 559–575.