Genetic Polymorphisms in Meningioma Formation and Progression

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Abstract

Sporadic meningiomas are common tumors of the arachnoid membrane that arise within the general population. Approximately half of these tumors arise through inactivation of the *NF2* tumor suppressor as determined by loss of heterozygosity (LOH) of 22q, the chromosome arm where the *NF2* locus is located. Recently, SNP309, a single nucleotide polymorphism in the promoter region of *MDM2*, was found to accelerate tumor progression by increasing levels of MDM2 protein, a negative regulator of p53. This study investigated a cohort of 92 sporadic meningioma patients and found that in those with both SNP309 and LOH of the *NF2* locus, 45.5% had high grade tumors compared to only 13.6% of those who retained both 22q alleles ($p = 0.019$). In those with LOH of 22q but not SNP309, males had significantly higher tumor grade ($p = 0.022$). However, among those with LOH of 22q and SNP309, gender and tumor progression were independent, ($p = 1.00$). This suggests SNP309's masking of the gender differentation in meningioma progression. This study established the prominence of the p53 pathway in meningioma progression and proposes a molecular model supported by the results.

1 Introduction

Meningiomas are tumors that arise from the arachnoid membrane [1], the lining of cells covering the central nervous system. They are mainly slow growing and make up about 20% of all primary intracranial tumors, with an incidence of about 6 cases per 100,000 of the population per a year [2]. Approximately 90% of meningiomas are benign tumors [3]. Nevertheless, more than 25% of them cannot be removed due to their inaccessible location. These benign tumors are left to enlarge [4]. In fixed spaces, such as the skull, the tumor begins to press upon the tissue of neurons around it. Excessive pressure interferes with normal function and can disable a critical nerve. For example, if a tumor compresses the optic nerve, vision could be lost. One way to better counteract meningiomas is to diagnose patients early, while the tumors are still inconsequential. Early identification would allow for more time to apply appropriate treatment to the meningiomas and attenuate the tumors before they become debilitating. Determination of specific populations at risk for meningiomas allows for an approximation of an individual's susceptibility. Appropriate monitoring may then be implemented.

Symptoms of ailments are usually apparent at the molecular level much earlier than they can be felt by the patient or diagnosed by doctors via contemporary imaging methods such as magnetic resonance imaging (MRI). The molecular factors behind tumor formation may be simple activation or inactivation events, or complex interplays. Activation of a proto-oncogene or inactivation of a tumor suppressor gene directly induces a cell to divide more often. However, most molecular pathways, involve feedback loops where a molecular pathway's products affect the original molecule itself. Often, many loops of intricate autoregulatory feedback interact so that a slightly different level of a gene's expression may also cause a significant change in a cell's mitotic activity.

Meningiomas arise in three genetic backgrounds: as sporadic tumors in genetically nor-

mal individuals, as multiple meningiomas, and as part of the neurofibromatosis 2 syndrome. This study focuses on sporadic meningiomas and all mention of meningiomas from here on can be assumed to refer to sporadic meningiomas. Many genetic events have been found to be associated with meningiomas in humans, such as inactivation of the *neurofibromatosis 2* (*NF2*) tumor suppressor, the common deletion polymorphism of *GSTT1*, and loss of *DAL-1* expression [5, 6, 7]. In particular, inactivation of *NF2* has been strongly correlated with meningioma initiation in about half of all cases [8]. Susceptibility to inactivation of *NF2* is determined by loss of heterozygosity (LOH) of 22q, the chromosome *NF2* is located on. Discovering such associations allows for development of additional meningioma diagnostic procedures and treatments. As part of Human Genome Epidemiology, an evolving field of inquiry that applies an epidemiologic approach to studying the human genome, genetic correlations can be used to design genetic tests that may predict an otherwise healthy person's risk of developing aggressive meningiomas. Preventive treatments may then be instituted in response [9], thereby improving the healthcare of those affected.

Differences in genes among the human population take the form of polymorphisms, where no allele is found at a frequency higher than 99%. Most polymorphisms are silent and have no differential effect. Polymorphisms may be of significance if they occur within genes that are expressed to create protein products. Other relevant polymorphisms occur within regions that promote or inhibit a certain gene's expression, affecting the levels of the gene's protein product. Many alterations in gene expression arise from single nucleotide polymorphisms (SNP), which involve only one nitrogenous base pair. SNP309 is located at the 309th nucleotide in the first intron of the *MDM2* promoter and has been associated with quickened tumor formation. In the general population, SNP309, a thiamine to guanine single nucleotide polymorphism, has a homozygous frequency of 12% (g/g) and a heterozygous frequency of 40% (t/g) [10]. Comparison to genotype frequencies in meningioma patients can reveal possible associations between SNP309 and meningioma formation or progression.

Transcription proteins increase the expression of certain genes. Sp1 is a universal transcription protein that binds to certain DNA sequences. The existence of SNP309 creates an additional region for Sp1 to bind and promote *MDM2* transcription [10]. The subsequent protein product is a ubiquitin ligase that binds directly to the active domain of p53, a crucial tumor suppressor that responds to intracellular stresses and prevents abnormal cell growth. The binding of MDM2 immediately inactivates p53, whose activity depends mostly on binding to DNA to engage mechanisms that repair or kill off abnormal cells. The resulting p53-MDM2 complex silences the genes that p53 would normally activate [11]. Then, the p53-MDM2 complex translocates to the cytoplasm, where it is degraded and cut up by the ubiquitin-proteosome system [12].

SNP309

↓

greater Sp1 affinity
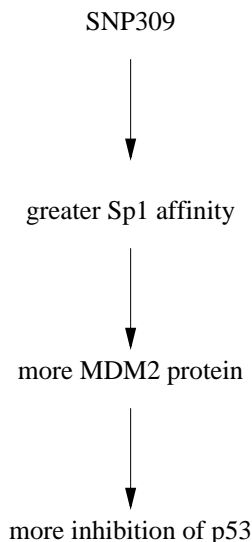
↓

more MDM2 protein

↓

more inhibition of p53

Figure 1: The Effect of SNP309: The occurrence of SNP309 in the *MDM2* promoter leads to inhibition of the tumor suppressor p53.

Because SNP309 increases the transcription of the proto-oncogene *MDM2*, the occurrence of SNP309 attenuates p53 tumor suppression and is associated with accelerated tumor formation (Figure 1). Clinically, this association has been confirmed for such cancers as soft tissue sarcomas, breast cancer, and osteosarcomas [10]. Merlin, the *NF2* protein prod-

uct, affects cell-cell contact inhibition. It has been established that the loss of this tumor suppressor initiates meningioma formation. In a previous study, merlin was found to also mitigate the inhibitory effects of MDM2 on p53. Since inactivation of the *NF2* gene is seen in approximately half of all meningiomas [8], changes in the activity of the p53 pathway due to SNP309 can be analyzed in tumors with and without *NF2* loss, providing an important model for understanding merlin function in the p53 pathway in human tumors. By using epidemiological methods to find the frequency of SNP309 among meningiomas patients, this paper explores the contribution of SNP309 to the risk of meningioma initiation and progression in a cohort of 92 sporadic meningioma patients. On a broad scale, this study contributes to the wealth of genetic information that will help improve the health of those affected by meningiomas.

2 Materials and Methods

2.1 Study Population

Between August 2000 and December 2004, 142 sporadic meningioma patients had tumors resected at the Massachusetts General Hospital (MGH). Matching blood and tumor samples were donated for research and stored in the MGH Neuropathology Tumor Bank. From the 142 patients, 50 were further excluded from the study due to lack of adequate blood or tumor DNA. A total of 92 patients were included in this study.

Blood and tumor samples were collected during the surgery. Blood DNA was extracted from the lymphoblast cells of the blood using a Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN). This study was approved by the Institutional Review Board of the Massachusetts General Hospital (MGH) and informed consent was obtained from all subjects.

The Research Patient Data Registry (RPDR), a database of clinical information from the

MGH, was used to obtain demographic and diagnostic data about each patient. Such data includes the gender, ethnicity, and age of patients, along with the tumor grade and location. This data was used to help search for any significant associations. In addition, previous study by Dr. Fabio Nunes used tumor samples to determine the loss of heterozygosity (LOH) of 22q among the study cohort. This too was used in analysis of the data.

2.2 SNP309 Genotyping

Polymerase Chain Reaction (PCR), a method of making numerous copies of a desired segment of DNA, was used to amplify the amount of the DNA sequence of interest as to facilitate analysis. In PCR, the primers determine the segment of DNA to be replicated. The primers P901 and P902 were designed and used to amplify a 209 bp (base pair) product (Figure 2). The PCR was carried out in a total volume of 10μL containing 100ng DNA, 4 pmol of both forward and reverse primers, 0.2 mM of each dNTP (nucleotides of each nitrogenous base), 2μL of $5\times$ Q-solution (Qiagen, Valencia, CA), 1μL of $10\times$ Buffer (provided by the manufacturer), and 0.25 U of Taq polymerase. The initial denaturation was carried out at 95°C for 4 min. This was followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 57°C for 30 sec, and DNA extension at 72°C for 1 min. Each complete cycle theoretically results in twice the amount of DNA.

After PCR, a restriction enzyme digest was used to facilitate the detection of SNP309. In the normal wild type PCR product, the enzyme *ApeK I* had two sites of cleavage. So, the 209 bp sequence was cut into three strands (35 bp, 68 bp, and 106 bp). The occurrence of SNP309 created an additional cleavage site so that four strands were created (35 bp, 11 bp, 57 bp, and 106 bp) (Figure 2). 10μL of PCR product was digested with the restriction enzyme *ApeK I* according to the manufacturer's specifications (New England BioLabs). The digest was then analyzed using an 8% polyacrylamide gel for electrophoresis, where strands of DNA travel through a gel matrix at rates dependent on their sizes. An AmpliSize^{TM}

WT

primer 901   primer 902

cgggagttcagggtaaaggt...14bp...ggctgcggggccgcttcg...50bp...cgctgc...80bp...tccgaaactgcactaaaagg

                              ApeK I   *ApeK I*

SNP309

primer 901   SNP309   primer 902

cgggagttcagggtaaaggt...14bp...ggctgcggggccgctgcg...50bp...cgctgc...80bp...tccgaaactgcactaaaagg

                              ApeK I   *ApeK I*   *ApeK I*

cleavage site
↓   WT   SNP309
ApeK I : g ' cwgc (w = a or t)   35 bp + 68 bp + 106 bp = 209 bp   35 bp + 11 bp + 57 bp + 106 bp = 209 bp
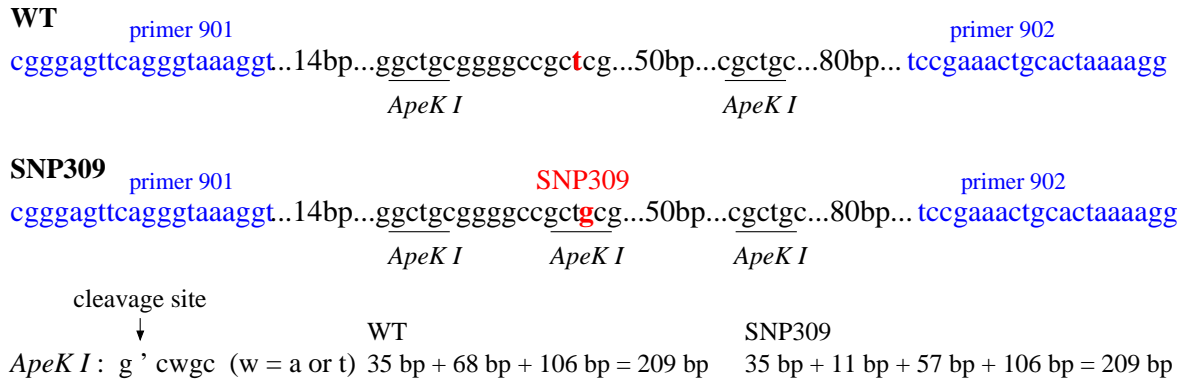
Figure 2: Theory Behind the Assay: Wild type PCR products have two sites of *ApeK I* cleavage. The 209 bp sequence is cut into three strands (35 bp, 68 bp, and 106 bp). The occurrence of SNP309 creates an additional cleavage site so that the PCR product is cut into four strands (35 bp, 11 bp, 57 bp, and 106 bp).

Molecular Ruler (BioRad, Hercules, CA) set of DNA strands of known lengths allowed for approximate determination of the length of DNA in the sample.

DNA sequencing was carried out to confirm that this method of genotyping was indeed correct. PCR product from a homozygous wild type (t/t), a heterozygous individual (t/g), and a homozygous SNP309 (g/g) were purified using the Qiagen PCR purification kit. 10μL of purified DNA was added to 10μL of 4.0×10^{-12} M primer. A different solution was created with both the forward primer (P901) and the reverse primer (P902) in order to increase the confidence in the sequencing results. Since there were two primers and three different genotypes, a total of six solutions were sent to the automated sequencing Core at MGH, where DNA sequencing was carried out by using Applied Biosystems Taq DyeDeoxy Terminator cycle sequencing kits. This method utilizes a fluorescently-labeled dideoxy-nucleotide chain method, where each base was fluorescently labeled with a different color. After cycle sequencing and clean-up, the DNA samples were put through capillary electrophoresis on an ABI 3700 PRISM automated sequencer. Fluorescent signals were then translated into corresponding base pair sequences.

2.3 Statistical Analysis

The Fisher's Exact test was used to test for significant associations between the SNP309 genotypes and clinical and demographic characteristics of the study patients. The p level for significance was set at $< .05$, and all tests were 2-tailed.

3 Results

3.1 Population Characteristics

This study included 92 meningioma patients. Of the study subjects, 83 were Caucasian (90.2%), 2 were of Asian decent (2.2%), and 2 were of African decent (2.2%) (the ethnicities of the remaining 5 were not recorded). There were 27 males and 65 females, with ages ranging from 29 to 83. Interestingly, only 65 (70.7%) of the patients had benign tumors, while 23 (25.0%) had atypical tumors and 4 (4.3%) had malignant tumors.

3.2 SNP309 Genotype Detection

In the genotyping of SNP309, occurrence of the 68 bp strand indicated the wild type allele (t), while the 57 bp strand indicated the SNP309 allele (g). If both strands were found in a sample, then the genotype was heterozygous and had one wild type allele and one SNP309 allele. Figure 3 shows an example of a gel of each genotype.

3.3 SNP309 in the Cohort

The homozygous frequency of SNP309 in the cohort was 15.3% (14/92) and the heterozygous frequency was 45.7% (42/92). This was not significantly different from frequencies in the general population (12% and 40%) ($p < 0.50$). The genotype frequencies did not differ much
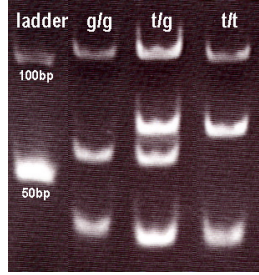
Figure 3: Gel of Digested PCR Product for Detecting SNP309 Genotype: The first and last band in each lane appear in all digested samples and serve as indicators that the digestive enzyme indeed carried out its function. Of the two middle bands, the higher one is 68 bp and indicates the wild type allele (t). The lower one is 57 bp and indicates the SNP309 allele (g). Presence of both bands means the DNA sample has both the t allele and the g allele.

when dividing the cohort by gender or age either (data not shown). The ethnic distribution was predominantly Caucasian, so other ethnicities had no statistical power.

3.4 Associations with SNP309

A summary of pertinent association is given (Table 1). Twenty-seven of the 42 individuals (64.3%) heterozygous for SNP309 (t/g) had LOH of 22q, while only 15 of 37 individuals (40.5%) with the wild type genotype (t/t) had LOH of 22q. When comparing those with heterozygosity of SNP309 to those without SNP309, occurrence of the SNP was significantly associated with LOH of 22q (p = 0.043). However, no results of significance were found when relating the homozygous state of SNP309 (g/g) to LOH of 22q.

Although there was also no significant relationship between the presence of SNP309 and tumor grade in our cohort of meningioma patients (p = 0.486), a specific subgroup of tumors, those with LOH of 22q, showed an interesting association between SNP309 and tumor grade. Among the 33 cases of those with both SNP309 and LOH of 22q, 15 (45.5%) had high grade tumors. Of the 22 individuals who had SNP309 but retained both *NF2* alleles, only 3 (13.6%) had high grade tumors. In the subgroup of individuals with SNP309, LOH of 22q

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	Subgroup	Association	p-value
(A)	meningioma patients	heterozygous SNP309 & 22q LOH	0.043
(B)	presence of SNP309	22q LOH & higher tumor grade	0.019
	presence of wild type	22q LOH & higher tumor grade	0.438
(C)	presence of 22q LOH & SNP309	men & higher tumor grade	1.00
	presence of 22q LOH & wild type	men & higher tumor grade	0.022

Table 1: Associations with SNP309: (A) There is significant linkage between heterozygous SNP309 (t/g) and LOH of 22q (p = 0.043). (B) In those with SNP309, LOH of 22q results in higher tumor grades. Without SNP309, there is no such correlation. (C) In those with both LOH of 22q and SNP309, there is no association between gender and higher tumor grade. In those with LOH of 22q but not SNP309, men have higher grade tumors.

was significantly associated with greater tumor progression (p = 0.019). In patients without SNP309, there was no significant correlation (p = 0.438).

Significant associations were also made in regard to gender. All 3 men who had LOH of 22q but not SNP309 had high grade tumors (2 atypical, 1 malignant). Meanwhile, 10 of the 12 women had benign tumors. In this subgroup, higher tumor grade was significantly associated with men (p = 0.022). In the subgroup of tumors with both LOH of 22q and SNP309, 8 of 18 women (44.4%) and 7 of 15 men (46.6%) had high grade tumors. Gender and tumor grade were independent (p = 1.00).

4 Discussion

4.1 The Study Population

The cohort group consisted of individuals who required removal of meningiomas. Though meningiomas are mostly asymptomatic, the cohort consisted of individuals who came to the hospital because they felt symptoms. Therefore, the study population consists of those with tumors more aggressive than in the general population of those with meningiomas. This explains the higher frequency of high grade tumors found in the study population (29.3%

versus 10% in general).

When looking at the frequencies of SNP309 genotypes in the study cohort, there was no difference from the frequencies in the regular population. In the study, SNP309 is not correlated with the general presence of meningiomas.

4.2 SNP309 in relation to *NF2*

A relationship was found between SNP309 and LOH of 22q, the chromosome containing the *NF2* tumor suppressor. In general, LOH of 22q is correlated with higher tumor grade. Looking at this association in the subgroup of those with SNP309 shows a similar significance. However, LOH of 22q was not correlated with high grade tumors in those with the wild type. This suggests that the general correlation between LOH of 22q and higher tumor grade arises from the stratification in those with SNP309.

4.2.1 SNP309 and *NF2* Molecular Interaction

Inactivation of *NF2* has been established as an initial step towards meningioma formation. *NF2* is a tumor suppressor gene, essential for cell-cell contact inhibition via the extracellular matrix [14]. Merlin, the product of the NF2 gene, was found to also mitigate the inhibitory effect of MDM2 on p53 [13]. In the 50-60% of those who gain meningiomas through loss of *NF2* activity, MDM2 levels may become elevated subsequently suppressing p53. Other proteins such as p14ARF also act to suppress MDM2 activity, but the absence of merlin may affect the balance of concentration levels, leaving the MDM2 levels more prone to increase. If individuals have SNP309 as well, MDM2 levels are naturally higher, and the meningiomas that form as a result of *NF2* inactivation will be more aggressive. Figure 4 shows a proposed pathway connecting SNP309 and merlin. Because the cohort in this study consists of individuals with more aggressive tumors, there must be selection for those with SNP309 among individuals with LOH of 22q. This was supported by the significant linkage

of heterozygous SNP309 and LOH of 22q in the study population. Though the homozygous state of SNP309 was not significantly correlated to LOH of 22q, enlarging the study cohort could change this. The low frequency of SNP309 in the general population resulted in only 13 individuals in this study with homozygous SNP309.

$$\text{SNP309} \longrightarrow \text{MDM2} \dashv \text{p53}$$
$$\text{Merlin}$$

Figure 4: The Interaction Between Merlin and SNP309: MDM2 acts as a proto-oncogene, suppressing p53 tumor suppression. Merlin, the *NF2* product, inhibits MDM2, preventing it from attenuating tumor suppression. When the *NF2* gene is inactivated, MDM2 becomes more active leading to meningioma formation. In individuals with SNP309, this effect is compounded, leading to more aggressive tumors.

4.2.2 SNP309, *NF2* and Gender Stratification

Previously, LOH of 22q was found to be correlated with higher tumor grade among men [15]. This study, in the subgroup of those with LOH of 22q but not SNP309 (homozygous wild type) also found a significant relation between higher tumor grade and men. However, there was no correlation in those with both LOH of 22q and SNP309. While meningiomas that arise because of *NF2* inactivation are more aggressive in men, the presence of SNP309 causes accelerated tumor progression without regard to gender. This suggests that the SNP309 mechanism of tumor progression is more pronounced than other pathways of meningioma progression since it masks the previously noted gender imbalance.

The reason for the gender imbalance among those without SNP309 has yet to be determined. Though presence of SNP309 masks the effects of gender on meningioma, discovering the origin of the gender bias would help in establishing whether the presence of SNP309 just masks the effects of that pathway or actually interferes with it.

4.3 Implication of SNP309 Significance

The assertion of merlin as a MDM2 regulator was confirmed clinically. For the first time in humans, merlin, the product of the *NF2* gene, was shown to be significantly associated with the p53 pathway.

The association of SNP309 with meningioma progression in those with LOH of 22q suggests inactivation of the p53 pathway as a prominent mechanism for meningioma progression. Studies and potential drugs targeting the p53 pathway can also be applied to the 50-60% of meningioma patients with LOH of 22q.

5   Conclusion

This study found significant association between SNP309 and tumor progression in patients with meningioma arisen from LOH of 22q. In addition, a proposed molecular pathway explaining the genetic correlations is clearly defined and supported. The significance of SNP309 suggests the prominence of p53 in meningioma progression.

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A   Abbreviations Used

MRI - magnetic resonance imaging

NF2 - neurofibromatosis 2

LOH - loss of heterozygosity

SNP - single nucleotide polymorphism

RPDR - research patient data registry

PCR - polymerase chain reaction

MGH - Massachusetts General Hospital

dNTP - deoxyribonucleotide triphophate

DNA - deoxyribonucleic acid

bp - base pair