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SHORT REPORT

Mutations in DNA polymerase η are not detected in squamous cell carcinoma of the skin

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The major etiological agent in skin cancer is exposure to UV-irradiation and the concomitant DNA damage. UV-induced DNA lesions, such as thymine dimers, block DNA synthesis by the major DNA polymerases and inhibit the progression of DNA replication. Bypass of thymine dimers and related lesions is dependent on the translesion polymerase DNA polymerase η (Polη). In the inherited disorder, xeroderma pigmentosum variant (XPV), inactivation of Polη results in extreme sensitivity to UV light and a marked increase in the incidence of skin cancer. Here, we tested the hypothesis that somatic mutations and/or polymorphisms in the *POLH* gene that encodes Polη are associated with the induction of UV-dependent skin cancers. We sequenced the exonic regions of the Polη open reading frame in DNA from 17 paired samples of squamous cell skin carcinoma and adjacent histologically normal tissue. We analyzed approximately 120,000 nucleotides and detected no mutations in *POLH* in the tumors. However, we identified 6 different single-nucleotide polymorphisms, 3 of them previously undocumented, which were present in both the tumor and paired normal tissue. We conclude that neither mutations nor polymorphisms in the coding regions of *POLH* are required for the generation of human skin squamous cell carcinoma.

Key words: squamous cell skin carcinoma; DNA polymerase-η; single nucleotide polymorphism; UV irradiation; xeroderma pigmentosum variant

Skin cancer is the most frequently detected cancer in humans. It is estimated that 1 of 7 people will develop some form of skin cancer in their lifetime.[1] With the exception of malignant melanoma, surgical excision of these malignancies is nearly always curative. However, the resulting disfiguration and cost of treatment are significant. The major etiological agent in skin cancer is exposure to UV irradiation, and the consequent introduction of thymine dimers and related lesions in DNA that block replication.[1,2] These lesions, if not repaired, can cause mutations. Important mechanistic clues to the induction of mutations by UV can be found in a rare inherited disease, xeroderma pigmentosum (XP). Individuals with XP have a defect in the repair of UV dimers and exhibit a 1000-fold increase in the incidence of all types of UV-induced skin cancers. Frequently, these persons succumb to malignant melanoma.[3] A subgroup of individuals [xeroderma pigmentosum variant (XPV)] has no defects in the removal of UV damage in DNA, but instead fails to copy past UV-induced lesions during DNA replication.[4] Bypass of thymine dimers in normal individuals is catalyzed by DNA polymerase-η (Polη), a translesion DNA polymerase that inserts the complementary nucleotides in newly synthesized DNA, and thereby promotes suppression of UV-induced toxicity and mutagenesis. Similar to other XP individuals, XPV patients have a higher frequency of skin cancers, presumably due to a lack of functional Polη.[5,6] Mutations that alter the splicing of Polη mRNA or reduce the expression of the protein have been identified in XPV.[7]

It has been suggested that somatic mutations or polymorphisms in Polη may reduce bypass activity and increase the likelihood that UV damage leads to induction of skin cancers.[8] Somatic mutation in Polη could be an early event in skin cell carcinogenesis and could be clonally present in the tumors. We tested the hypothesis that somatic mutations and/or polymorphisms in the Polη gene are associated with sporadic skin cancer. Our approach was to directly sequence the Polη gene, *POLH*, in squamous cell carcinomas and adjacent normal skin in order to identify single nucleotide polymorphisms (SNPs) and mutations that might lead to reduced bypass efficiency.

Materials and methods

Patients and tumor samples

Candidates were recruited from among Caucasian immunocompetent individuals, 40–80 years of age, from the King County area of Washington State, receiving their primary care from a University of Washington Medical Center physician for at least the 5 previous years. Cases of both genders were recruited from a pool of subjects with suspicious skin lesions, but no prior incidence of squamous cell skin cancer or history of other cancers. Written consent was obtained from all participating patients. Biopsies were procured from both the site of the suspicious tissue lesion and from tissue proximal to the lesion.

PCR amplification and DNA sequence analysis

Genomic DNA was prepared from tumors and adjacent histologically normal tissue using a DNeasy Tissue kit (Qiagen, Valencia, CA). Primers for PCR amplification were designed based on the works of Yuasa *et al.*[9] and information was obtained from the relevant exon–intron borders maps using Sequencher 4.2. The sequences of the primers and the locations in the *POLH* gene are indicated in Table I. Using approximately 50 ng of genomic DNA as a template, and both the forward and reverse primers, first-round PCR amplification was performed with an Expand High-Fidelity PCR kit (Roche, Palo Alto, CA), according to the instructions provided. Amplification was performed on exons 2–11; exon 1 is upstream of the translational start site and was not analyzed here. One microliter of product from the first round of amplification was used as a template in a second round of PCR amplification (50 μL), using nested forward and reverse primers in the same protocol. The nested primers were similar to that for first-round amplification but contained 2–5 additional, complementary nucleotides at the 3′-end. The PCR products were separated and visualized by electrophoresis in a 1.5% agarose gel, and then purified using a Montage PCR96 Cleanup Plate kit (Millipore) and subjected to direct sequencing with an automated DNA sequence analyzer, at the University of Washington Biochemistry Sequencing

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Facility. Sequences were analyzed using Sequencher 4.2. All presumptive changes in nucleotide sequence were confirmed both by sequencing the opposite DNA strand and by repeating the entire PCR procedure.

Reverse transcriptase PCR amplification

RNA samples were prepared from tumors and adjacent normal tissue using an RNeasy Mini kit (Qiagen, Valencia, CA). Using approximately 100 ng of RNA as a template, and the primers indicated in Table I, a region including exons 2–5 was amplified as a single segment with an Access Reverse Transcription PCR system (Promega, Madison, WI). An aliquot (1 μL) of the RT-PCR amplified product was subjected to a second round of PCR amplification with nested primers. An aliquot of the amplified product was analyzed using electrophoresis in 1.5% agarose gel for changes in length.

TABLE I – SEQUENCE OF PRIMERS USED FOR PCR AMPLIFICATION OF THE EXONIC REGIONS OF THE HUMAN *POLH* GENE

Amplicon	Primer
Exons 2–3	
F2	CTGGCATTTTGGATTAGGTG
R3	TTGACTCAGCCTTTCCTGG
Exon 4	
F4	GCAGACAATCTCAAGGTTGC
R4	CAGGAAGCAGAAGCAGTATG
Exon 5	
F5	GGTCTTCTAAGCTGGCTGCC
R5	ACATAGGCGTGCGAAACTGC
Exon 6	
F6	TGGGGATGTTGTGGGATACC
R6	TATGTATTTCCCCTGGCTC
Exons 7–9	
F7	TCCTGAACCTTTTGGAG
R9	TTGTTCCTGCTCCATAAGG
Exon 10	
F10	GTACCTAGAATTCAGATGCTCC
R10	CAGAGCTAAGAATCATCATCTATTC
Exon 11	
F11	CATCTATGGATTAAATCTGTCC
R11	TGAAGAGATGGGACCGTAAC
RT Exon 2–5	
F1	GTTTTGCCAGGTGTTTGTTACC
R5	ATCAATCTGAAGAGAATCGAGCC

Results

In our previous work, we used random mutagenesis to identify Polη mutants with impaired enzyme activities.[10] Using a yeast model system, we showed correlation between impaired polymerase activity and sensitivity to UV radiation. Our *in vitro* and *in vivo* results are in accord with the hypothesis of McCulloch *et al*. that mutations in Polη could be associated with sensitivity to UV radiation and to skin tumors in normal UV-exposed individuals.[8] To test this hypothesis, we examined the nucleotide sequence of the coding region (exons 2–11) of the Polη gene in DNA from 17 samples of squamous cell carcinoma. DNA encoding Polη from adjacent histologically normal tissue was sequenced whenever a nucleotide substitution was identified in the tumor sample. We amplified the exons, including the indicated flanking introns, as illustrated in Figure 1, using the stated PCR-primer pairs. Exons 2 and 3, together with the intervening intron, were amplified as a continuous segment comprising 1239 base-pairs; for this amplicon only, we sequenced DNA from an additional 31 histologically verified squamous cell skin carcinomas. Exons 7–9 were amplified as a continuous segment together with the introns between exons 7–8 and 8–9. In total, we carried out about 300 different sequencing reactions and analyzed approximately 120,000 nucleotides. We detected a total of 6 heterozygote single-base substitutions in DNA from the tumor samples; no deletions or insertions were observed. None of the substitutions were tumor-specific. Each was also present in DNA from the adjacent normal tissue, indicating that the observed substitutions were in fact SNPs.

The 6 SNPs we observed are shown in Figure 1 and Table II. Three are novel, and 3 have been previously documented in tissues from normal individuals. The previously known SNP E2+276

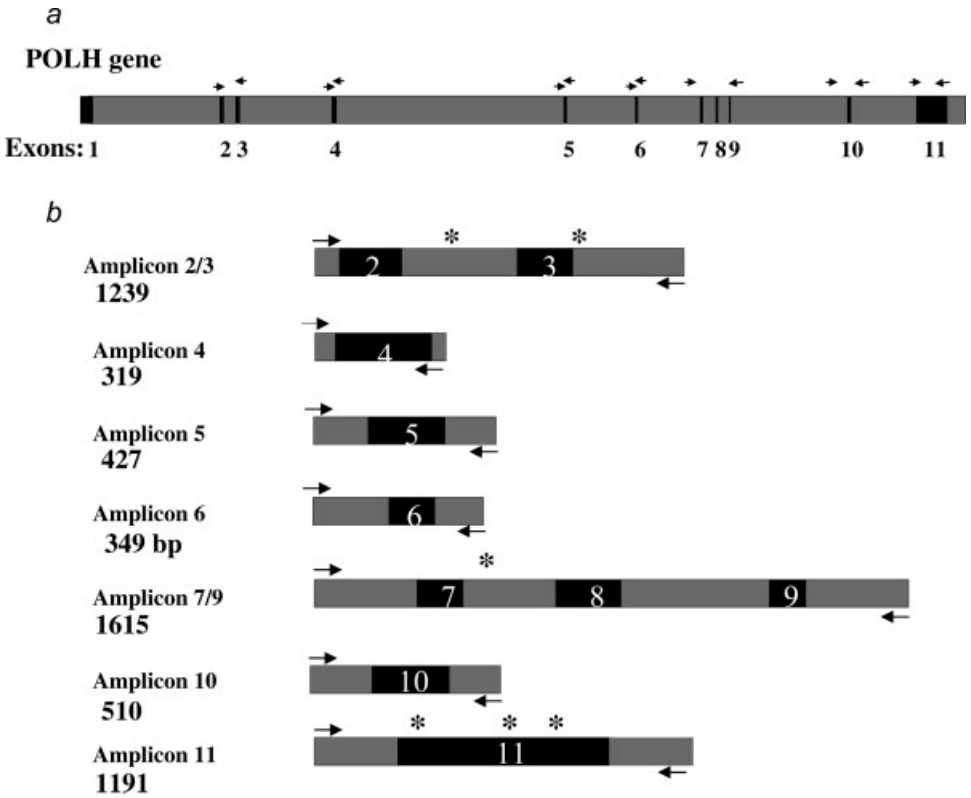

FIGURE 1 – Schematic illustration of (*a*) the locations of the primers in the *POLH* genomic region, (*b*) the PCR amplicons that were sequenced. The nucleotide changes are indicated. Black color indicates an exonic region. The location of Primer (→) and SNPs (*) are indicated.

TABLE II – POLη SNPS IDENTIFIED IN SQUAMOUS CELL CARCINOMA DNA

SNP location	SNP reference	Sequence
E2+276	RS9333508	AGAGATGGGGATTGGTTGG **G/A** GGGGCAGGGCTCACTA
E3+4		AAGCTAACCTCACCAAGTA **A/G** GAAAAAAACATTATTT
E7+360		GTGGCTTGCTTGGCATTTT **T/C** CCAAAAATACAAATGA
E11 G1434A T481T		CCACTAAGAAAGCAACCAC **G/A** TCTCTGGAATCATTCT
E11 A1783G M595V	RS9333555	TAAAGCAACTCCTGCAGAG **A/G** TGGATTTGGCCCACAA
E11 A1939T M647L	RS6941583	CCTGGTACCGGTATGGGAT **A/T** TGCCAGAACACATGGA

This is an illustration of the locations of SNPs. The exon names, nucleotide and amino acid changes are indicated. Gray color indicates exonic regions.

(RS9333508), which we identified in 3 of 48 tumors, is a G to A transition located in intron 2, at a position 276 bp distant from the exon–intron border. SNP E7+360, identified in 3 tumor/normal sample pairs, is a T to C transition located 360 bp beyond the exon–intron border in intron 7 and has not previously been described. SNP E3+4, which we detected in one sample pair, is an A to G transition located at the third nucleotide in intron 3, immediately following exon 3. This SNP is in the sequence that participates in the splicing process,[11] and thus could be significant for proper splicing of Polη mRNA. We therefore performed RT-PCR on tumor RNA. We found no evidence of abnormally spliced transcripts (data not shown), suggesting that the splice site has retained flexibility and is not altered by the G to A substitution. We did not observe this SNP in the 31 additional samples of squamous cell carcinomas that we screened; thus, it is unlikely to occur at a frequency of more than 1% in the general population.

We identified three different SNPs in exon 11; Synonym SNP, G1434A, resulting in no amino acid change. Nonsynonym SNP RS9333555 (A1783G), resulting in a methionine to valine substitution at position 595 (Met595Val). And a nonsynonym SNP RS6941583 (A1939T), resulting in a methionine to leucine substitution at position 647 of Polη (Met481Leu). Interestingly this SNP is in a conservative residue that is located within the putative zinc finger domain of Polη. The effect of this substitution on the enzyme function *in vivo* should be evaluated.

Discussion

Patients with XPV have a high frequency of skin cancers presumably due to a lack of functional Polη. All Polη mutations that have been identified in XPV result in impaired catalytic activity. Based on the sunlight sensitivity of XPV patients, the low fidelity of Polη, and the types of DNA sequence alterations found in skin tumors from normal individuals, McCulloch *et al*. hypothesized that mutations in Polη could be associated with skin tumors in normal, UV-exposed individuals.[8] In this work, we tested the hypotheses that: (1) *de novo* clonal mutations in the gene encoding Polη are found in squamous cell carcinoma of the skin, and (2) polymorphisms in Polη that could be predicted to result in changes in protein function are present at high frequency in patients with squamous cell carcinoma of the skin. Upon extensive DNA sequencing, we found no Polη mutations in 17 samples of squamous cell skin carcinoma and paired normal tissue. Our results lead to the conclusion that mutations in the coding region of Polη are not necessary for the development of cutaneous squamous cell carcinoma. However, our results cannot exclude the possibility that mutation in control regions of the *POLH* gene might affect the level of expression of Polη. Additionally, translesion DNA polymerases other than Polη have the potential to bypass UV-induced DNA lesions. Polι and Polκ have been shown to copy past DNA lesions *in vitro*,[12] and Polκ has been found to be overexpressed in several human cancers.[13–15] Hence, mutations in these genes, or overexpression, could have a role in skin carcinogenesis.

We observed 6 different SNPs in the *POLH* gene, 3 of them novel. One of the new SNPs is located at a splice site, but we found no evidence of aberrant RNA splicing. Currently, 6 nonsynonymous SNPs have been identified in *POLH*, two of them present in our samples. The frequencies of the known SNPs are between $<1\%$ and 15% and most are in partially conserved amino acids. The possible biological significance of nonsynonymous SNPs should be considered in light of the dramatic effect of impaired Polη function in XPV patients. It is conceivable that one or more of these SNPs causes a small change in the function of Polη, and that a large-scale population study will be required to determine if a specific SNP alters the incidence of UV-induced skin cancers. Regardless, the absence of clonal somatic mutations that we observed here provides evidence that mutations in Polη are not an early event in squamous cell carcinoma in the skin.

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