Early-onset of sporadic basal-cell carcinoma: Germline mutations in the TP53, PTCH, and XPD genes

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Background: **Basal cell carcinoma (BCC) is the most common non-melanoma skin cancer in the Western world. The objective of this study was to examine together germline mutations in the TP53, PTCH, and XPD genes as risk factors for developing BCC at a young age. We hypothesized that mutations in these genes significantly increase the risk of early-onset BCC (£35 years).**

Methods: **The PCR, DNA sequencing and Restriction Fragment Length Polymorphisms methods were utilized to study eight Puerto Rican patients with a confirmed diagnosis of BCC before age 35.**

Results: **A novel germline mutation (T:A transversion) was identified at the exon 4, codon 50 of the TP53 gene of one BCC patient. No other mutations were found at the TP53 or PTCH genes. The presence of the XPD mutant allele is associated with a seven-fold increase in risk (OR=7.0, p=0.03) for developing BCC prior to age 35. In addition, the DNA Repair Capacity (DRC) of these BCC patients showed a 47% reduction that was significant in relation to age-matched controls (p=0.021). However, the XPD mutant allele was not associated with the decrease in DRC observed in BCC participants.**

Conclusions: **The evaluated population presented BCC before age 35, a phenomenon that is so rare, as to make very difficult the study of this subpopulation with a larger sample size. The results of this study, suggest that the XPD Lys751Gln polymorphism may have a significant role in the development of early-onset BCC in the Puerto Rican population.**

Key words: TP53, XPD, DNA repair capacity.

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most common skin cancers in the Western world collectively termed non-melanoma skin cancer (NMSC) (1, 2). Prolonged exposure to environmental ultraviolet (UV) light, skin type, age, gender, and DNA repair capacity (DRC), are well known risk factors associated with NMSC (3, 4, 5, 6, 7, 8). Previous studies have concluded that mutations in genes that function as tumor suppressors, cell cycle regulators, and nucleotide excision repair play an important mechanistic role in the development of NMSC. These types of gene categories are well represented by the TP53 (tumor suppressor and cell cycle regulator), PTCH (tumor suppressor) and the XPD (nucleotide excision repair) genes.

The p53 protein (encoded by the TP53 gene) known as "the guardian of the genome", acts mainly as a tumor suppressor but it also functions as a transcription factor in the cell cycle control and regulates many important pathways including DNA repair. The frequency of mutant p53 alleles in human tumor cells isolated from various organs ranges from 6-48% (IARC, release R11, 2006). In terms of skin cancer, more than 50% of human tumors show a TP53 mutation with a range from 30-50% in persons with BCC (7, 9, 10). Similarly, mutations in the human homologue of the *Drosophila* patched gene (PTCH) have been identified in 20-54% of the sporadic BCC cases (7, 11, 12, 13). The PTCH gene is a tumor suppressor gene that acts in opposition to the Hedgehog signaling protein, controlling the cell polarity, segmentation, and growth rate in numerous tissues. The PTCH mutations are also responsible for causing the Gorlin syndrome, a condition whose main clinical feature includes the development of skin tumors at an early age (14, 15). Importantly, over 50% of the PTCH and TP53 gene mutations identified from NMSC tumors include the UV-molecular signature of C to

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Funding Source: RCMI Program, NCRR, NIH to Ponce School of Medicine # 2G12 RR03050-17

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T and CC to TT transitions (13, 16, 18, 19). These lesions have been commonly associated with UVB radiation received by sun exposure. Over 60% of the BCC's studied from persons with Xeroderma Pigmentosum (XP) show mutations in the PTCH gene, in addition to the typical nucleotide excision repair (NER) pathway gene mutations. XP patients have at least 1,000-fold higher risk for developing skin tumors (20) and also develop skin tumors 2 to 3 decades earlier in comparison with the general population.

Several large-scale case-control studies suggested that a decrease in DRC, particularly in the NER pathway is a risk factor for development of NMSC (3, 6, 8). In a study of the Puerto Rican population, the average decline in DRC in NMSC (in relation to controls without skin cancer) has been reported to be approximately 42% (6). A decrease in DRC is associated with a lower capacity for repairing (removing) the UVB-induced DNA damage and predisposes the individual for the development of NMSC (6). One of the genes in the NER pathway is the XPD (ERCC2). This gene encodes for an ATP-dependent 5'-3' DNA helicase, responsible for opening the DNA around the UV-caused damage. A relationship between ERCC2 and TP53 in the development of skin tumorigenesis has been previously hypothesized (21, 22, 23), based on the physical and functional interactions of the ERCC2 (as a transcription factor) with the p53 protein, through the TFIIH-mediated complex. This coincidental association may represent the link between p53, the NER, transcription, and the cell growth regulation mechanisms, which contributes to the complex network of molecular events leading to genetic instability and skin tumorigenesis.

The incidence of sporadic BCC increases after 55 years of age with the highest incidence reported in individuals who are 70-years or older. Although BCC is rare in people younger than 40 years of age, in recent years, there has been an increase in the incidence of BCC among this group (24, 25, 26). This increase in BCC cases among young people has been attributed to changes in the general population behaviors resulting in an increase in outdoors activities**.** However, only a few studies have focused on this particular young subpopulation as a group of interest (7, 27, 28) and only one study examined for specific somatic mutations in both the TP53 and PTCH genes (7). Because of its geographical location (18º 15' N and 66º 30' W), Puerto Rico has registered one of the highest UV indexes (>8) recorded within 58 cities within the US and its territories (http://www.cpc.ncep.noaa.gov/products/stratosphere/uv_index/uv_annual.shtml). This study represents the first effort to examine together germline mutations in the TP53, PTCH, and XPD genes as a risk factor for developing BCC at a young age. We initially hypothesized that mutations in these genes significantly increase the risk of early-onset BCC (younger than 35 years) and may partially explain the occurrence of this disease in young persons. We have also evaluated the effect of mutated alleles in terms of their potential impact on the defects in DNA repair previously reported for the Puerto Rican population (6).

Materials and Methods

Studied Population

The Internal Review Board of the Ponce School of Medicine approved the use of Human Subjects. Informed consent was obtained from all participants prior to their enrollment. A survey of our database with 474 NMSC patients and 313 controls gathered as part of a seven year case-control study in Puerto Rico led to the selection of eight persons who developed BCC prior to age 35. These patients did not have a diagnosis of Xeroderma pigmentosum (XP) or any other genetic defects associated with an impairment of DNA repair capacity. The occurrence of BCC at such relatively young age is unusual enough for it to be considered "early-onset" and is reflected in the small sample size of eight early-onset cases recruited from 474 NMSC cases. Due to the fact that DRC declines with year of age, and for statistical purposes, the eight patients were age-matched with eight controls without skin cancer. All participants were Puerto Ricans with age ranging 21 to 35 years. All BCC participants had histopathologically confirmed BCC. A board-certified dermatologist examined the participants without skin cancer to exclude the presence of skin cancer or any pre-malignant lesion(s). Each participant voluntarily completed a seven-page questionnaire that solicited detailed information regarding other skin cancer risk factors.

DNA Extraction and PCR

An aliquot of five million peripheral lymphocytes per participant was used to extract DNA, utilizing the Wako DNA Extractor WB kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). We used the TP53 gene primers for amplification of exons four to eight (4-8), as described by Zhang, *et al.* (7). The PTCH gene exons 5,8,9,13,14 and 15 were amplified using the primers sequences previously described by Xie, *et al*. (29) and for the XPD (Lys751Gln) we employed the primer sequences described in Baccarelli, *et al.* (17). All primers were modified with an M13 tail for Infra Red (IR) sequencing purposes. PCR reactions (50 µl) were prepared using 100 ng of DNA and the QIAGEN Hot Start Taq PCR Master Mix Kit (QIAGEN Inc., Valencia, CA), following the manufacturer's instructions. Positive and negative controls were included using reference human DNA (Promega Corp., San Louis, CA) and water,

respectively. The cycling conditions were set in a Stratagene RoboCycler (Agilent Technologies Co., La Jolla, CA) as follows: 95°C for 15 min, 35 cycles of 94°C for 30 sec, 45 sec at correspondent annealing (17, 29, 30) and 72 °C for 1 min. This was followed by a final extension cycle of 72°C for 10 min. Amplification was confirmed by 3% agarose gel electrophoresis (90.0 V for 30 min), after which, amplified products were visualized under UV light.

Sequencing of TP53 and PTCH genes

Positive PCR samples were purified using the DNA Clean & Concentrator™-5 kit (Zymo Research Co., Orange, CA). The IR label M13 (IRD Dye-800-M12CF) was used as required for the sequencing in a LI-COR sequencer model 4300 (LI-COR Inc., Lincoln, NE). Sequencing protocols were performed following the manufacturer's specifications. Detected mutations were confirmed by a PCR and sequencing reactions' duplicates using the correspondent original DNA as the template.

XPD RFLP's and genotyping

The confirmed positive XPD PCR products (324 bp) were further exposed to restriction fragment length polymorphisms (RFLP's) by using the enzyme *Pst I*, as previously described in Baccarelli, *et al*. (17). The genotypes were determined by electrophoresis in a 3% agarose gel, by identifying the obtained band patterns as follows: A/A (wild-type homozygote): 224 and 100 bp; A/C (heterozygote): 224, 100, and 66 bp; and C/C (mutant homozygote): 100, 158, and 66 bp. In order to validate the RFLP's genotyping, five PCR products were selected randomly and purified (DNA Clean & Concentrator™-5, Zymo Research, Orange, CA). These genotypes were confirmed by direct DNA sequencing using (IR) in a LI-COR sequencer model 4300 (LI-COR, Inc., Lincoln, NE), following the manufacturer's specifications. No ambiguities were observed in the genotypes obtained by both methods.

DNA repair capacity (DRC) assay

A detailed description and validation of the host-cell reactivation assay to measure DRC has been published previously (31). This assay was modified with the luciferase reporter gene as published by Ramos *et al.* (4) and Matta *et al*. (6).

Statistical Analyses

The allele frequencies were calculated for the Puerto Rican skin cancer-free population (n=178), for the eight participants with BCC, and for the eight, age-matched controls. Differences in DRC were compared by means of the Mann-Whitney test. Odd ratios (OR) with 95% Confidence Interval (CI) were calculated using SPSS 12.0.1(SPSS, Chicago, IL). Correlations for the presence of a mutant allele, DRC and skin cancer were calculated also with a 95% CI. The Hardy Weinberg (HW) equilibrium, which is an equation that describes the genetic balance within a certain population, was calculated for the 178 controls without skin cancer from Puerto Rico utilizing software available as public domain at http://www.changbioscience.com/genetics/hardy.html.

Results

This study examined the possible role of mutations at the TP53, PTCH, and XPD genes in the development of early-onset BCC before the age of 35. The mean age for both controls and BCC participants groups was 30.0 years.

Mutations found in the TP53 and PTCH genes

Patient C2 was found to be heterozygous for a germline mutation at the position 13360 (Exon 4, Codon 50) of the TP53 gene. Screening for the other participants (seven patients and eight controls), revealed no mutations in the TP53 gene. Mutation screening for the PTCH gene showed that none of the participants had mutations in the selected exons' regions (5, 8, 9, 13, 14, and 15) of the PTCH gene. These exons are frequent "hot spots", where most of the PTCH mutations have been reported.

XPD Lys751Gln polymorphism

Within the studied subpopulation (eight BCC patients and eight aged matched-controls) only one BCC patient was identified as a mutant homozygote (C/C) for the XPD Lys751Gln and one as a wild type homozygote (A/A) (Table 1). Approximately 62.5 % of the participants who developed BCC before the age of 35 were heterozygous (A/C). No homozygote mutants were found in the control group studied. The XPD Lys751Gln allele distribution in a sample of 178 skin cancer-free Puerto Ricans did not fulfill the conditions of the HW test (p=0.0034). The frequency of the C allele in the BCC patients was two times higher

Table 1. Genotypic frequency of the Lys751Gln XPD gene. The total number of participants per category is represented by "n"; BCC: Basal cell carcinoma; AA: wild type homozygote; AC: heterozygote; CC: mutant homozygote; Hardy Weinberg equilibrium (p=0.00034).

	Genotype			
	AA	**AC**	**CC**	**n**
Total Puerto Rican Controls	93	83	2	178
Controls (≤35 years old)	4	4	0	8
BCC (≤35 years old)	1	5	2	8

than age-matched controls or the skin cancer-free Puerto Rican population (Table 2). Strikingly, the presence of the XPD mutant allele represents a seven-fold increase in risk (OR=7.0, p=0.03) for the development of BCC at an early age (d" 35 years).

Table 2. Comparison of the Hispanic Puerto Rican (HPR) population XPD Lys751Gln allele frequencies with other ethnic groups. The number of total alleles is represented by "n"; the asterisk (*) represents allelic frequencies retrieved from the SNP500Cancer database (NIH National Cancer Institute) and for which skin cancer status is unknown; A: wild type allele; C: mutant allele.

		Frequencies	
Population	**n**	**A**	**C**
Hispanic-Puerto Rican Controls	356	0.76	0.24
HPR-controls (≤35 years)	16	0.75	0.25
HPR-NMSC (≤35 years old)	16	0.44	0.56
Hispanic*	46	0.65	0.35
African American *	48	0.83	0.17
Caucasian *	62	0.73	0.27

DCR analyses

The eight BCC patients studied had a statistically significant (p=0.021), reduction of DRC of 47% when compared to age-matched controls. However, the association between the XPD mutant allele and the decrease in DRC was not statistically evident (p>0.05). None of the eight BCC patients had a diagnosis of XP or any genetic condition associated with impairment of DRC.

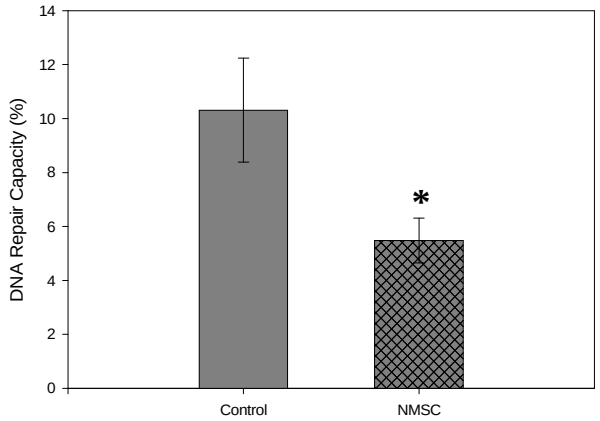

Figure 1. DNA repair capacity (DRC) of eight participants diagnosed with the Non-melanoma skin cancer (NMSC) basal cell carcinoma at 35-years of age or less. Compared to the DRC of eight age-matched controls without skin cancer, persons with NMSC had a reduction of 47% in DRC that was statistically significant; *:p ≤ 0.05)

Other Risk Factors

Although sample size was limited, we evaluated other risk factors that might influence the development of this disease at a young age. Within the studied variables, we found that having a family history of NMSC increases significantly the risk for early-onset BCC (OR=2.67, p=0.007) (data not shown). This data was retrieved from a seven-page questionnaire that was completed by the participants.

Comments

This study sought to examine for possible etiologic factors that might explain the occurrence of early onset BCC in the Puerto Rican population. This study also represents the first effort to examine together germline mutations in the TP53, PTCH, and XPD genes as a risk factor for developing BCC at a young age. Contrasting previous studies, which analyzed these alterations at the somatic level (tumors) (7, 16, 17, 18, 19), we analyzed germline mutations which are less commonly studied and that usually have major impact in the phenotype, if a further mutation occurs. Literature showed that we have identified a novel TP53 mutation that had not been previously reported as a TP53 hot spot for BCC (32) or other cancers (33). This mutation is an unusual T to A transversion, not commonly associated with damage caused by UV light. However, this was a silent mutation still coding for the amino acid Isoleucine at the exon 4, codon 50 of the TP53 gene. None of the eight cases studied had mutations in the selected exons of the PTCH gene. Because we focused only on germline mutations, more data from the Puerto Rican population BCC tumors is required. The obtained data will contribute to compare with other populations the impact and incidence of TP53 and PTCH mutations at the somatic level.

The presence of the XPD mutant allele found in this study is associated with a seven-fold increase in risk (OR=7.0, p=0.03) for the development of BCC at an early age (≤35 years), when compared with aged-matched controls. Although skin cancer is clearly a multifactorial process, it appears that the XPD mutant allele of this gene may increase the risk of early-onset BCC in the Puerto Rican population. Furthermore, these young persons carrying the mutant allele might have an increased risk of developing a second primary cancer either of skin or other organs (34). In addition, the XPD allele distribution did not fulfill the conditions of the HW test. This equation is used to describe the genetic balance within a particular population. This result is consistent with a recent study in albinism conducted by Santiago-Borrero, *et al*. (35), in which the total studied Puerto Rican population (n=229)

did not pass the HW test. The discrepancy between the observed and expected frequencies may be explained by the genetic heterogeneity of the Puerto Rican population where the founder effect or other genetic influence affects the HW criteria. Although the BCC patients showed to be in HW equilibrium, because of the limited sample size (eight), the HW test should not be applied to the BCC subpopulation studied.

The observed 47% reduction in DRC is consistent with a previous study, which employed the same methodology for DRC analyses and showed an average reduction of 42% in the DCR of NMSC patients (6) but XPD allelic frequency was not determined for this group. The average age of onset for BCC patients in Puerto Rico was reported as 66 years (6). Our results contrasted a previous study, in which it was found that cells carrying the mutant homozygous genotype (C/C) showed only a marginally lower ability to reactivate the UV-irradiated plasmid (36). However, this study was not stratified within the same age parameters, using 40 years as the cut-off age. The function of the XPD protein in these BCC young patients was expected to be responsible for affecting the DRC through the NER helicase function. Unexpectedly, the association test for the presence of the XPD mutant allele and the DRC did not show statistically significant difference. These results suggest that the XPD mutant allele might be predisposing to skin cancers due to an altered interaction within the cell cycle control pathway, rather than through reduced NER, as previously suggested (37). This study provides new molecular insights related to the genetic predisposition of Puerto Ricans for developing BCC at an early age.

Resumen

Trasfondo: El carcinoma de célula basal (CCB) es el cáncer de piel no-melanómico más común en el mundo occidental. El objetivo de este estudio, fue examinar mutaciones germinales en los genes TP53, PTCH y XPD como factores de riesgo para desarrollar CCB a temprana edad. Hipotetizamos que mutaciones en estos genes aumentan significativamente el riesgo de padecer CCB antes de los 35 años. ***Métodos:*** Los métodos de PCR, secuenciación de DNA y patrones generados por restricción enzimática en polimorfismos ("Restriction Fragment Length Polymorphisms") fueron utilizados para estudiar ocho pacientes puertorriqueños con un diagnóstico confirmado de CCB antes de los 35 años de edad. ***Resultados:*** Una nueva mutación germinal (transversión T:A) fue identificada en el exón 4, codón 50 del gen TP53 de un paciente con CCB. No se encontró ninguna otra mutación en los genes TP53 o PTCH. La presencia del alelo mutante de XPD fue asociado con un aumento en riesgo siete veces mayor, para desarrollar CCB antes de los 35 años (OR=7.0, p=0.03). Además, los pacientes con CCB mostraron una reducción de 47% en su capacidad de reparación del DNA (CRD) la cual fue significativa en comparación a controles de la misma edad (p=0.021). Sin embargo, el alelo mutante de XPD no fue asociado con la disminución en CRD observada en los participantes con CCB. ***Conclusiones:*** La población evaluada presentó CCB antes de los 35 años; un fenómeno tan raro que hace muy difícil estudiar esta particular subpoblacion con un tamaño de muestra mas amplio. Los resultados obtenidos en este estudio sugieren que en la población puertorriqueña, el polimorfismo Lys751Gln del gen XPD podría tener un papel significativo en el desarrollo de CCB a temprana edad.

Acknowledgements

This project was supported by 2G12 RR03050-17 RCMI Program, NCRR, NIH to Ponce School of Medicine. Technical support was provided by the PSM Molecular Genetics Core Laboratory under the RCMI Program. We also appreciate the technical contribution of Dr. A. Ingraffea (PSM), Mr. R. Ortiz and Mr. J. Cobian (UPR-Ponce) and the assistance for manuscript editing provided by Mrs. H. Delgado (PSM) and UPR-Ponce faculty (Biology and Chemistry Departments and Deanship for Academic Affairs).

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