Published in final edited form as:
Int J Cancer. 2014 January 1; 134(1): . doi:10.1002/ijc.28339.

Allele-specific imbalance mapping identifies *HDAC9* as a candidate gene for cutaneous squamous cell carcinoma

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Abstract

More than 3.5 million non-melanoma skin cancers were treated in 2006; of these 700,000 were cutaneous squamous cell carcinomas (cSCC). Despite clear environmental causes for cSCC, studies also suggest genetic risk factors. A cSCC susceptibility locus, *Skts5,* was identified on mouse chromosome 12 by linkage analysis. The orthologous locus to *Skts5* in humans maps to 7p21 and 7q31. These loci show copy number increases in approximately 10% of cSCC tumors. Here we show that an additional 15-22% of tumors exhibit copy-neutral loss of heterozygosity. Furthermore, our previous data identified microsatellite markers on 7p21 and 7q31 that demonstrate preferential allelic imbalance (PAI) in cSCC tumors. Based on these results, we hypothesized that the human orthologous locus to *Skts5* would house a gene important in human cSCC development and that tumors would demonstrate allele-specific somatic alterations. To test this hypothesis, we performed quantitative genotyping of 108 single nucleotide polymorphisms (SNPs) mapping to candidate genes at human *SKTS5* in paired normal and tumor DNAs. Nine

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Brief Description: Human chromosome 7p21 shows evidence of preferential allelic imbalance (PAI) and copy neutral loss of heterozygosity in cutaneous squamous cell carcinomas (cSCCs). 7p21 is orthologous to a mouse skin cancer susceptibility locus, *Skts5*. Candidate genes at *Skts5* identified from the mouse were assessed for evidence of PAI in human cSCCs. Multiple variants in *HDAC9* were identified that show evidence of allele-specific gains in cSCC. These data suggest that *HDAC9* may be important in cSCC development.

Conflict of Interest
K. Ridd is an editor at Nature Publishing Group.

SNPs in *HDAC9* (rs801540, rs1178108, rs1178112, rs1726610, rs10243618, rs11764116, rs1178355, rs10269422, and *rs12540872*) showed PAI in tumors. These data suggest that *HDAC9* variants may be selected for during cSCC tumorigenesis.

Keywords

HDAC9; cutaneous squamous cell carcinoma; allelic-specific imbalance; *Skts5*

Introduction

Non-melanoma skin cancer (NMSC), consisting of cutaneous basal cell carcinoma and cutaneous squamous cell carcinoma (cSCC), is the most common cancer in the world. More than 3.5 million NMSCs were treated in 2006; of those, approximately 700,000 were cSCCs [1]. From 1994-2006, a greater than three-fold increase in skin cancer incidence has been observed [2]. There are several environmental risk factors for cSCC; the best documented is ultraviolet radiation [3]. Family and population-based studies also suggest the role for inherited risk factors for cSCC [4,5], but there are few well validated genetic risk variants.

Multiple groups have identified skin cancer susceptibility loci using mouse models [6]. In one study, thirteen skin tumor susceptibility (*Skts*) loci were identified by linkage analysis between skin tumor resistant, *Mus spretus*, and skin tumor susceptible, *Mus musculus*, mice [6-8]. About 40% of susceptibility loci identified in these crosses showed preferential allelic imbalance (PAI) in tumors, indicating that allele-specific somatic alterations provide another approach to map susceptibility loci [9]. Studies in human tumors demonstrate the presence of preferential allelic imbalance (PAI) for putative cancer susceptibility variants as well [10,11].

7p21 and 7q31 are the human orthologous regions to a mouse cSCC susceptibility locus, *Skts5,* on mouse chromosome 12[8]. Our lab previously conducted array comparative genomic hybridization (aCGH) on 305 skin tumors including 222 cSCCs. 10% of tumors showed gains of chromosome 7. Additionally, we performed a study of preferential imbalance using 270 tumors, some overlapping with the aCGH tumor set, from 65 individuals and identified nine loci showing evidence of preferential allelic selection in tumors. Two regions on chromosome 7 showing evidence of PAI,were 7p21 and 7q31[12]. As more samples than expected showed allelic imbalance on 7, in this study, we looked at the 59 tumors that overlapped between experiments for evidence of copy-neutral loss of heterozygosity (LOH).

Based on our evidence from both mouse models and human tumors that *SKTS5* may house a skin cancer susceptibility allele, we hypothesized that variants conferring risk for human cSCC on chromosome 7 would show PAI in tumors. We identified candidate genes for evaluation in human samples based on potentially functional sequence variations and differential gene expression between the mouse strains used for linkage [8]. We chose tagging single nucleotide polymorphisms (SNPs) for PAI studies in human cSCCs for our two strongest candidates, aryl hydrocarbon receptor (*AHR*) and histone deacetylase 9 (*HDAC9*), and SNPs in or near seven additional candidates, cadherin-related family member 3 (*CDHR3*), B-cell associated protein 29 (*BCAP29*), synaptophysin-like protein (*SYPL*), Ets variant 1 (*ETV1*), diacylglycerol kinase-β (*Dgkβ*), interferon-related developmental regulator 1 (*IFRD1*), and anterior gradient 2 (*AGR2*), for PAI in human cSCCs. These studies collectively highlight the importance of investigating allele-specific alterations in tumors to identify risk regions and variants.

Materials and methods

Copy-neutral LOH study

Using previously generated data, detailed in Dworkin et al, 2010[12], we compared the aCGH profiles and microsatellite genotyping data from 59 tumors.

Human samples for imbalance studies

This study was approved by the OSU Institutional Review Board. All study participants signed informed consent. Normal genomic DNA (blood) and available matched cSCCs were collected from 156 individuals ascertained from dermatology and transplant clinics. Criteria for study inclusion was a medically confirmed diagnosis of cSCC, availability of a normal source of DNA and a source of tumor DNA, pathology records, and completion of a questionnaire detailing sun-exposure, cancer, and immunosuppressive history. To ensure that multiple tumors from the same individual were not clonally related, we used tumors from different anatomical locations. Re-excisions were not included. Tumors were stained with hematoxylin and eosin and reviewed by pathologists. Areas containing greater than 70% tumor cells were microdissected from formalin-fixed paraffin embedded (FFPE) tissue sections.

DNA isolation

Tumor DNA was isolated from FFPE tissue containing at least 70% tumor cells as described [12]. DNA from blood samples was extracted by the OSU Human Cancer Genetics Sample Bank.

Allele-specific imbalance studies

We conducted quantitative genotyping of matched normal and cSCC tumor DNA pairs using Sequenom MassARRAY Iplex gold genotyping technology according to the manufacturer's protocol (Sequenom)(San Diego, CA). 481 independent tumors from 156 patients were included for study. Selection of genes for analysis was based on our *Skts5* mouse data [8]. Tagging SNPs for *AHR* and *HDAC9* were genotyped, along with a single coding SNP for seven additional candidate genes for a total of 108 SNPs (Supporting Information Table 1). Tagging SNPs were selected using the International HapMap Project Tagger program with a pairwise method of calculation with a 0.8 cutoff value for coefficient of determination (r^2) and a 0.2 cutoff value for minor allele frequency in Caucasians. TagSNP details are included in Supporting Information Table 2. Genotypes of poorer quality and those which had strong calls in a water samples were eliminated from analysis. Duplicate tumor and matched normal DNA samples, control samples with known genotypes, and no template controls were included as controls. An allelic imbalance ratio (R) to measure imbalance for each normal/tumor pair was calculated as described [12]. Chi-square tests were used to detect PAI for each SNP. *p*-values were corrected using the Bonferroni method [13].

Results

Copy-neutral LOH at SKTS5

Previous array comparative hybridization (aCGH) studies of cSCC showed gains of 7p21 and 7q31 corresponding to human *SKTS5* in about 10% of samples[12]. Two of nine microsatellite markers from these regions, D7S503 and D7S2418, demonstrated statistically significant evidence of PAI (*p*-values of 0.047 and 0.016 respectively) [12]. As more samples than expected (>10%) from our microsatellite genotyping studies showed imbalance, we hypothesized that it was possible that copy-neutral loss of heterozygosity (LOH) was

occurring. To obtain evidence of copy-neutral LOH, we looked for samples that showed no copy number imbalance for chromosome 7 by aCGH, but showed imbalance when comparing normal and tumor DNA by microsatellite genotyping. From our previously generated datasets, we identified 59 tumors with both aCGH and genotyping data. We assessed these tumors for copy-neutral LOH. Of these 59 tumors, 39 tumors (66%) were concordant between the aCGH and genotyping data sets (i.e. both methods indicated copy number aberrations or both methods showed no evidence of copy number aberrations), while 20 tumors showed discordant results for copy number between aCGH and genotyping methods (i.e. aCGH showed no copy number aberrations and genotyping indicated imbalance or vice versa). Of the 20 samples which were discordant between the aCGH and genotyping methods, nine showed strong evidence of copy-neutral LOH. Four samples may have copy-neutral LOH, but as these had fewer than two informative (heterozygous) markers for the region we were unable to make a definite conclusion. The remaining seven samples showed copy number gains by aCGH, but showed no imbalance by microsatellite genotyping when using a cutoff for imbalance of a normal to tumor allelic ratio greater than 1.5 or less than 0.67. To determine the total frequency of imbalance by either aCGH or copy-neutral LOH, we combined our aCGH and microsatellite data for the 59 tumors. Six tumors (10%) showed copy number gains by aCGH that were confirmed by genotyping. Another nine tumors (15%) showed evidence of copy-neutral LOH by multiple markers (Table 1). Four tumors (7%) showed inconclusive evidence of copy-neutral LOH, meaning these samples had one marker showing imbalance, but had multiple markers for which we had no data (data not shown). Thus, we estimate the frequency of copy-neutral LOH for chromosomes 7 is between 15 and 22%. These results suggest that 25 to 32% of cSCC tumors exhibit genetic alterations for human *SKTS5* when both LOH and copy-neutral LOH are considered.

Preferential allelic imbalance studies of candidate genes

As our microsatellite data at human chromosome 7 showed evidence of PAI in cSCCs and this region correlates to a mouse linkage region for skin cancer, we hypothesized that human *SKTS5* houses a susceptibility allele for cSCC [12]. We previously generated sequence and expression data for the 65 genes and non-coding elements mapping to *Skts5* for strains of mice used for the linkage analysis [8,14]. Based upon our mouse data we identified candidate genes for evaluation in human cSCCs. Genes were chosen if they had amino acid differences between the susceptible and resistance strains of mice and/or showed significant strain-specific differences in mRNA expression by qPCR. Two of the strongest genes were *Ahr* and *Hdac9* because these had both amino acid differences and expression differences between the mouse strains [8] . We chose tagging SNPs in *AHR* and *HDAC9* and SNPs in or near seven additional candidate genes showing either expression differences, potentially functional coding variants and/or suspected contributions to cancer to analyze for PAI in cSCC tumors (Supporting Information Table 1). We genotyped a total of 108 SNPs in 481 independent tumors from 156 individuals (Supporting Information Table 1 and 2). Nine SNPs showed evidence of PAI, two of which, *rs801540* and *rs1178108*, were the most significant (uncorrected p-values <0.05) (Table 2). Thirty-eight tumors heterozygous for *rs801540* showed allelic imbalance; eight showed relative gain of the G-allele and 30 showed relative gain of the T-allele (p-value, 0.0004; Bonferroni adjusted p-value, 0.0377) (Table 2). Of the 25 heterozygous tumors showing allelic imbalance for *rs1178108,* 6 showed relative gain of the A-allele and 19 showed relative gain of the G-allele (p-value, 0.0093; Bonferroni adjusted p-value, 0.9788) (Table 2).

Discussion

This is the first study, to our knowledge, that performed high-density PAI studies of a human ortholgous locus for a mouse tumor susceptibility region. These data confirm our previous studies in which markers on chromosome 7 demonstrated PAI [12] and complement the mouse linkage studies [6-8]. Our data showed eight *HDAC9* SNPs with suggestive evidence and one *HDAC9* SNP with statistically significant evidence for PAI in cSCCs, indicating these variants or those they tag for could represent variants important in the development or progression of cSCC.

Utilizing previous aCGH and microsatellite data, we identified 15-22% of tumors demonstrating copy-neutral LOH at *SKTS5* which provides additional support for this region as being important in cSCC development. LOH is commonly evaluated by measuring changes in copy number; however LOH can also occur independently of copy number change. Copy-neutral LOH has been observed in multiple types of cancers [15,16]. Studies that use copy number changes to identify candidate loci may overlook regions with high levels of copy-neutral LOH. Here, we correlated aCGH and microsatellite genotyping data. We identified 9 of 59 tumors with copy-neutral LOH at *SKTS5*, suggesting a higher frequency of imbalance at this locus than previously appreciated and increasing the potential relevance of this locus in cSCC.

This study identified nine SNPs within *HDAC9* which demonstrated PAI in cSCCs, suggesting this gene may play an important role in skin cancer. HDAC9 is a class IIa histone deacetylase family member and is thought to regulate the epigenetic status of histones by catalyzing deacetylation. In the strains of mice used for mapping *Skts5*, we identified both potentially functional amino acids and differential expression of *Hdac9* [8]. Aberrant *HDAC9* expression is observed in several types of cancers including medulloblastoma [17], acute lymphoblastic leukemia [18], and cervical carcinoma [19]. *HDAC9* has non-histone protein targets including forkhead box protein 3 [20], ataxia telangiectasia group D-complementing protein [21], and glioblastoma 1 protein [22]; which are members of pathways implicated in tumorigenesis [21,23]. Inhibition of *HDAC9* also inhibits cellular proliferation and induces apoptosis [21, 22]. Genomewide association studies have identified SNPs in *HDAC9* that are associated with male-pattern baldness [24]. Taken together, these studies suggest a link between *HDAC9*, the skin/hair follicle and cancer-related phenotypes.

Alleles showing preferential gain in tumors or those that they tag for may be strong candidates for risk association studies. Gain of specific alleles in tumors may indicate growth or selective advantage of cells containing these alleles. We do not yet know which SNP is the “causal” SNP driving the observed PAI. The nine *HDAC9* SNPs showing PAI in tumors are intronic, as are the majority of SNPs for which that they tag. If the causal SNP is one from our study, they may be influencing splicing, regulation through enhancer activity or noncoding RNA, or affecting the methylation status of *HDAC9*. Preferential gain of *HDAC9* variants in tumors is suggestive that the gained alleles may act to promote cancer phenotypes. These variants may induce stronger expression and/or activity of HDAC9, as both characteristics have been linked to tumorigenesis.

There are limitations to this study. We only assessed variants from a limited number of genes at *SKTS5* and may have missed the causal gene or variants. Genes were prioritized based on the mouse data, but only two genes were represented by multiple tagging SNPs. We have strong coverage for both *AHR* and *HDAC9*, although not all SNPs or haplotypes may be fully represented. Because we only analyzed one tagging SNP in the other genes, these genes were not comprehensively evaluated. We also only chose SNPs with a high degree of heterozygosity as we were not adequately powered to detect preferential allelic

imbalance for variants with low heterozygosity frequencies; thus this study is underpowered for the detection of rarer SNPs. Another important consideration is that only one of the SNPs showing evidence for preferential allelic imbalance met multiple comparisons adjustments for the 108 SNPs evaluated. However, as many of these SNPs are highly correlated and are in linkage disequilibrium, a conservative Bonferroni correction may not have been an appropriate method for adjustment. It is possible that the SNPs identified in this study are playing a role in tumorigenesis and may be somatic targets. Future studies will focus on identifying the causal SNP for the observed PAI and functional studies *in vitro* to characterize the variants driving imbalance and their potential role in cancer initiation and progression.

In summary, this study highlights the importance of investigating copy-neutral LOH to identify loci critical for tumor development. Furthermore, our data support the use of cross-species strategies to identify candidate genes. We identified *HDAC9* as a candidate gene for human cSCC using a combination of linkage mapping in the mouse with targeted PAI mapping in human tumors. Although there is strong evidence for PAI for *SKTS5* and *HDAC9*, additional functional, genetic, and population-based studies are necessary to follow up on these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the OSU CCC Nucleic Acids Research Shared Resource for genotyping assistance, the OSU CCC Tissue Procurement Shared Resource for ascertainment of tumor samples, and the Human Genetics Sample Bank for sample preparation. Dr. David Lambert and Ms. Ilene Lattimer were instrumental in identification of patients for study. OSU Control specimens were provided by the OSU Human Genetics Sample Bank. We thank Charles Toland for development of a Java script used for Sequenom data analysis.

Financial Disclosure

This work was supported by the American Cancer Society [grant number RSG-07-083 MGO to A.E.T.], the National Institutes of Arthritis and Musculoskeletal and Skin [to B.C.B.], the National Cancer Institute [grant number CA134461 to A.E.T], the Ohio State University Comprehensive Cancer Center; and an Up on the Roof Fellowship and an Alumni Grant for Graduate Research and Scholarship [to J.F].

Abbreviations

cSCC	cutaneous squamous cell carcinoma
HDAC9	histone deacetylase 9
NMSC	non-melanoma skin cancer
PAI	preferential allelic imbalance
SNP	single nucleotide polymorphism
AHR	aryl hydrocarbon receptor
CDHR3	cadherin-related family member 3
BCAP29	B-cell associated protein 29
SYPL	synaptophysin-like protein
ETV1	Ets variant 1

Dgkβ	diacylglycerol kinase-β
IFRD1	interferon-related developmental regulator 1
AGR2	anterior gradient 2
aCGH	array comparative genomic hybridization
LOH	loss of heterozygosity

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Table 1

cSCC tumors showing copy-neutral LOH for SKTS5

Samples	SQ26.3	SQ36.10	SQ36.6	SQ46.8	SQ52.4	SQ52.5	SP51	SQ29.1	SQ38.8
aCGH	NI	NI	NI	NI	NI	NI	NI	NI	NI
Markers									
D7S503	ND	I	ND	ND	ND	ND	H	I	ND
D7S1818	I	I	ND	NI	H	H	H	I	NI
D7S644	ND	H	H	ND	H	H	H	ND	ND
D7S1799	ND	ND	ND	ND	ND	ND	I	ND	ND
D7S2420	H	ND	ND	ND	I	I	ND	ND	ND
D7S2418	ND	H	H	H	H	H	ND	H	NI
D7S486	I	I	I	I	H	H	H	H	I
D7S1873	ND	I	I	I	H	H	ND	NI	I

LOH, loss of heterozgyosity; aCGH, copy number data as measured by array comparative genomic hybridization for chromosome 7; I, Imbalance; NI, No imbalance; ND, No data; H, Homozygous for microsatellite marker.

Table 2

SNPs showing preferential allelic imbalance in cSCC tumors

SNP	Location	Gene	Genotype	Location	Allele 1	Allele 2	*p*-value
rs1178355	18204811	*HDAC9*	G/T	INTRON	11	3	0.0325
rs10243618	18522740	*HDAC9*	A/G	INTRON	7	1	0.0339
rs1726610	18630208	*HDAC9*	G/T	INTRON	16	5	0.0163
rs801540	18672384	*HDAC9*	G/T	INTRON	8	30	0.0004*
rs1178108	18743747	*HDAC9*	A/G	INTRON	6	19	0.0093
rs1178112	18746213	*HDAC9*	A/G	INTRON	3	13	0.0124
rs11764116	18800413	*HDAC9*	G/T	INTRON	11	1	0.0039
rs12540872	18836667	*HDAC9*	G/A	INTRON	24	11	0.0280
rs10269422	18854601	*HDAC9*	T/A	INTRON	3	12	0.0201

Allele 1, number of heterozygous tumors showing relative gain of allele 1; Allele 2, number of heterozygous tumors showing relative gain of allele 2; *p*-values are unadjusted for multiple comparisons testing

*significant after Bonferroni correction for 108 SNPs.