Frequent Loss of Heterozygosity Targeting the Inactive X Chromosome in Melanoma

James O. Indsto, Najah T. Nassif, Richard F. Kefford, and Graham J. Mann

ABSTRACT

After previous preliminary observations of paradoxical deletion events affecting the inactive X chromosome in melanoma, we have surveyed the X chromosome for deletions using 23 polymorphic microsatellite markers in 28 informative (female XX) metastatic melanomas. Ten tumors (36%) showed at least one loss of heterozygosity (LOH) event, and in two cases an entire chromosome showed LOH at all informative loci. Four distinct X chromosome smallest regions of overlap can be resolved. An 18.6-Mb region on the p arm involving 9 of 28 (32%) samples lies between the markers DXS1061 and DXS1068. An equally frequently deleted smallest region of overlap straddled the centromere, bounded by DX1024 on the p arm and DXS983 14.6 Mb away in Xq11–12. One tumor potentially defines this region more tightly to a 10.6-Mb smallest region of overlap bounded by DXS1190 and DXS987 that contains the androgen receptor (AR) gene. A 6.2-Mb deleted region can be defined between the markers DXS8051 and DXS9902 in 8 of 28 (28%) tumors. An additional, less frequently deleted region of 25.7 Mb was found on distal Xq between the markers DXS1212 and DXS1193 in 5 of 28 (18%) tumors. X inactivation analysis of five tumors with LOH, using the AR exon 1 CAG repeat, showed that in each case, the inactive, hypermethylated allele was the one deleted. Analysis of copy number in this region by quantitative PCR showed restoration to disomy and, in one case, trisomy at AR.

INTRODUCTION

Chromosomal deletions and amplification events are frequent in advanced neoplasms and provide evidence for the existence and location of putative tumor suppressor genes (TSGs) and oncogenes. In melanoma, 9p and 10q deletions are most frequent, and losses on 6q, 11q, 1p, 15p, 17q, and 18q are also frequently found (1, 2). However, no studies have focused on the X chromosome. In the human female, random inactivation of one copy of an X chromosome by hypermethylation is an early event in embryogenesis, resulting in most tissues being a mosaic in terms of X inactivation status. Neoplasms, being clonal expansions of single precursor cells, usually share a common X inactivation status. This can be conveniently assayed using the methylation-sensitive restriction enzyme HpaII, which has a recognition site within the highly polymorphic exon 1 CAG repeat (ARTR) in the androgen receptor (AR) gene.

We previously examined X inactivation status in a cohort of common benign nevi but found loss of heterozygosity (LOH), targeting the inactive X chromosome at ARTR, in 4 of 12 metastatic melanomas that had been included as positive controls for neoplasia (3). We therefore undertook to construct a deletion map of the X chromosome to locate putative TSG(s) or oncogene(s) relevant to melanoma and explain the paradoxical deletion events involving the AR region.

In recent years, numerous reports have implicated X chromosome deletions and amplifications in breast (4, 5), ovarian (6), prostate (7), and testicular cancer, with a putative tumor suppressor defined to Xq27 (8). The AR, as well as being a useful marker for X inactivation and clonality assays, has been directly implicated in a number of hormone-dependent neoplasms. Specific high-level Xq11–12 gains, which include AR, have been reported in metastatic prostate cancer (7). However, aberrant methylation of CpG islands in prostate cancer cell lines has also been reported to be associated with loss of AR expression (9). Loss of the active allele at ARTR, with, by implication, loss of AR expression, has been reported in female breast cancer (5). Studies of ovarian carcinomas report exclusive loss of the inactive chromosome. Cheng et al. (6) report frequent interstitial loss of Xq12, including AR, in low metastatic potential tumors. Choi et al. (10), in a study of advanced ovarian carcinomas, report frequent Xq25–26.1 deletions involving the inactive allele. In a study of borderline and invasive epithelial ovarian tumors, Edelson et al. (11) report a 1-cM region flanking AR as the most frequently deleted, whereas in borderline tumors, the nearby proximal locus DXS1194 was more frequently deleted.

In neoplasia showing LOH at ARTR, it is possible, by applying X inactivation analysis, to further determine whether the active or inactive gene copy is targeted by LOH. In the case of the active gene copy being targeted by LOH, digestion of the remaining tumor DNA with a methylation-sensitive restriction enzyme such as HpaII is expected to cause little further change in observed allelic ratios after PCR amplification of ARTR. This
is because the remaining tumor DNA of such a neoplasm with LOH will be hypermethylated and will not be susceptible to enzyme digestion. In the case of LOH targeting the inactive gene copy, a different pattern is expected to result. In this case, the remaining tumor DNA will consist of predominantly unmethylated DNA, and digestion with HpaII should result in degradation of all remaining tumor DNA. Any signal obtained from ARTR will be of markedly lower intensity, being due only to residual methylated stromal DNA which will generally show random X inactivation status. This, however, should not be assumed because stromal tissues may also show skewed X inactivation, with blood lymphocytes from elderly women being a particularly dramatic example (12).

In this study, we also carried out analysis of copy number at ARTR to shed light on the mechanism by which the inactive chromosome had been targeted. LOH data alone are insufficient to do this because a number of molecular mechanisms may give rise to a similar observed allelic shift (13). Chromosomal loss in tumors is frequently followed by replacement with duplication of the remaining allele. Alternatively, gene amplification events may produce an allelic imbalance that mimics LOH. Because both mechanisms are possible explanations of our findings, we undertook gene dosage analysis using a multiplex PCR method based on that of Poropat et al. (14).

MATERIALS AND METHODS

DNA Samples. Twenty-nine sporadic metastatic melanoma samples from female patients were used in this study. Tumors were surgically excised, snap frozen, and stored at −70°C. Peripheral blood samples were also taken from all patients to establish constitutional status and stored at −20°C. DNA extraction from blood and tumor tissue was undertaken by standard methods (15). DNA was diluted to 20 ng/µl with TE and stored at 4°C for PCR amplification.

LOH Analysis. Twenty-three microsatellite markers spanning the whole X chromosome were studied with markers designed to provide a comprehensive deletion map (see Fig. 1). Marker locations were checked using the National Center for Biotechnology Information and the University of California at Santa Cruz Biotechnology human genome portals. Primer sequences were obtained from the Genome Database. PCR primers were purchased from Sigma-Genosys, (Australia), fluorescent forward primers were 5'-HEX-labeled.

PCR conditions were as described previously (16), except for the addition of BSA. High BSA concentrations in PCR reactions overcome melanin inhibition (17), so we included BSA at 1.2 µg/µl and successfully amplified even heavily melanin-contaminated tumor DNA. After amplification, an equal volume of denaturing sample dye (formamide containing 10 mM EDTA (pH 8.0) and bromphenol blue) was added, then the samples were heat denatured (95°C, 3 min) and snap chilled on ice. An aliquot (2 µl) of each sample was loaded onto a 6% (29:1) polyacrylamide gel containing 0.6× Tris-borate EDTA and electrophoresed (900V, 40°C) in 0.6× Tris-borate EDTA on a gel scanner (Catalogue No. 901, Amersham). Product sizes were determined using TAMRA-labeled GeneScan-350 standards (Applied Biosystems).

Scoring of LOH. Computer software provided with the Gel-Scan 2000 gives real-time plots of signal voltage versus gel retention time. The package allows analysis to determine product size and the area under the curve, which corresponds to product yield for LOH calculations. We used the formula of Cawkwell (18) with a change in allelic ratios of >30% in duplicate samples scored as LOH. This criterion is designed to permit LOH detection in tumor samples with up to 50% stromal contamination. Additionally, we have distinguished samples that showed LOH with an allelic change of >50% from those that showed a less dramatic allelic shift in the 30–50% range. Weaker LOH values may have a number of possible causes, such as stromal contamination and genetic heterogeneity of the tumor tissue sampled. Flagging of such samples with weaker LOH values (represented in lowercase) gives a measure of robustness of the deletion map.

X Inactivation Analysis. For X inactivation analysis, the test DNA from tumors with LOH at AR was split into an untreated aliquot and another aliquot subjected to digestion with the methylation-sensitive restriction enzyme HpaII (Promega Corp.). Digests were conducted at 37°C overnight, and the enzyme was heat inactivated (95°C, 10 min) before PCR of the ARTR locus. Relative allelic signal ratio before and after digestion of both normal and lesion tissue was compared. The X inactivation assay algorithm is analogous to that used for LOH analysis, and a >30% change in allelic ratio in at least duplicate samples of undigested and HpaII-digested tissue DNA was predetermined to indicate that the remaining chromosome was in fact the active X.
Gene Dosage Determination. In this assay, we sought to study whether in female patients with metastatic melanoma, LOH at the ARTR locus occurred via a mechanism that resulted in a change in gene dosage of the retained chromosomal copy. The protocol is based on the published method of Poropat et al. (14). Two single copy genes (target and reference) were amplified by PCR: a 463-bp product comprising exon 4 of the AR gene was coamplified with a reference sequence [283 bp comprising exon 2 of the β-globin (BG) gene located on 11p and unlikely to be deleted in melanoma]. The BG exon 2 forward primer used was 5′-CTCTGCCTATTGCTATTTTCCC-3′, and the reverse primer used was 5′-GAAACATCAAGGGTC-CCATAGAC-3′. The PCR was initiated by denaturation (95°C, 3 min) followed by 28 cycles of denaturation (95°C, 30 s), annealing (60°C, 45 s), and extension (72°C, 45 s), followed by a final extension of 72°C for 10 min. PCR reactions were performed as quintuple replicates in a 10-μl final volume and contained 20 pmol of each primer, 0.2 mm each deoxynucleotide triphosphate, 1 unit of Taq DNA polymerase (Amersham), proprietary buffer adjusted to a final MgCl2 concentration of 2.5 mm, 1 μg/μl BSA (17), and 5 ng of DNA sample. PCR reactions included healthy male and female control samples, and each tumor sample was matched against corresponding constitutional DNA. Male and female healthy control reactions were included on each gel. A one third volume of nondenaturating loading dye containing bromphenol blue was mixed into each sample, and 2 μl were loaded onto a 5% (29:1) nondenaturing polyacrylamide gel and electrophoresed in 0.5 x Tris-borate EDTA (700 V, 35°C) on a Corbett Gel-Scan 2000 DNA Analyzer, which allows quantitative analysis of PCR product yields in a manner analogous to that used in LOH analysis.

Statistical Analysis. The ratio of target:reference (AR/BG) PCR product yield for five replicates of male and female control samples and five replicates of malignant melanoma samples (along with constitutional DNA pairs) was studied. For each set of five replicate samples, the following statistics were determined: mean; SD; and 95% confidence interval (95% confidence interval, 1.96 SD/√n). The population mean and 95% confidence bandwidth were also determined for all female control samples.

RESULTS

LOH Analysis. Fig. 2 shows a deletion map of the X chromosome obtained using 23 polymorphic microsatellite markers in 29 female subjects with metastatic melanoma. One (tumor 1082) was homozygous for all markers examined, presumably due to Turner syndrome, and is not counted among informative tumors below. Ten (10 of 28, 36%) tumors showed LOH of at least one marker. Six of these showed LOH in more than one region, whereas LOH was found at all informative loci in two tumors. The most frequent losses were found at DXS1061 at Xp21–22 (7 of 20 tumors, 35%), DXS991 and DXS1190 at Xp11 [6 of 19 tumors (31.6%) and 6 of 18 tumors (33.3%)], and DXS1053 at Xp22 (4 of 17 tumors, 23.5%).

The X chromosome is functionally monosomic, so each observation of LOH has a 50% probability of causing a complete knockout of local gene functions in the absence of other compensatory changes in copy number or gene activation. For autosomal genes, this is much less likely. The observed rate of LOH (35%) effectively makes the X chromosomal genes in these regions among the most frequently targeted in melanoma.

Four smallest regions of overlap of deletions could be resolved on the X chromosome in females with metastatic melanoma. Three tumors (tumors 1079, 1380, and 1915) define an 18.6-Mb region between the Xp markers DXS999 and DXS1068 with 9 of 28 (32%) of samples implicated. An equally frequent smallest region of overlap straddled the centromere, being bounded by DXS11204 on the p arm and DXS983, 14.6 Mb telomeric in Xq11–12. In tumor 1079, the total signal from marker DXS1190 was reduced to a level 10–20% of that observed in the flanking markers DXS991 and DXS1213, both of which showed LOH. DXS1190 is therefore very likely to be homozygously deleted in that tumor. One tumor (tumor 1942), bearing a single deletion in this region, potentially further defines the smallest region of overlap to a 10.6-Mb interval between DXS1190 and DXS981. An additional Xp smallest region of overlap involving 8 of 28 (28%) tumors can be defined between markers DXS8035 and DXS9902. This region is defined by the tumors 1079 distally and 1380 proximally. Tumor 1088 contains an Xp deletion that straddles these smallest regions of overlap. Inclusion of this information potentially further reduces the Xp smallest region of overlap to either the interval between DXS987 and DXS9902 distally or between DXS999 and DXS1061 proximally. Finally, 5 of 28 tumors define a 25.7-Mb region on distal Xq defined by the markers DXS1212 proximally and DXS1193 distally.

Deletion and X Inactivation at AR. The AR gene lies within the pericentromeric smallest region of overlap defined by DXS1190 and DXS983, though it was not included in the homozygous deletion bounded by DXS991 and DXS1213. Six of 22 informative samples studied for deletions at ARTR showed LOH (22.7%), and X inactivation analysis was carried out on five of these samples. The remaining sample (tumor 1088) showed marginal LOH with only 35% average allele in X inactivation analysis. This outcome is compared with predicted allele ratio and allele ratio relative to undigested DNA. Additionally, where one allele was reduced by the LOH event, the remaining allele was always reduced in the X inactivation assay. This indicates that the hypermethylated, inactive allele had been deleted by the LOH event in each case, a result with only a 3% probability of occurring by chance.

One of these samples (tumor 1163) is illustrated in Fig. 3; it shows a shift in allelic ratios of ~80% due to LOH and a similarly large reduction of the remaining active, unmethylated allele in X inactivation analysis. This outcome is compared schematically with the expected profile that would be obtained for a sample with LOH of the active allele. In such a case, tumor DNA, contributed by the active, unmethylated allele would already be reduced by LOH and would no longer be available for enzyme digestion in the X inactivation assay. As a consequence, little change would be expected in allelic ratios in tumor DNA both before and after the X inactivation assay.

Gene Dosage Determination. To determine the mechanism of LOH targeting the inactive chromosome in the AR region, we conducted gene dosage analysis at AR and BG using a quantitative PCR assay (Fig. 4). AR/BG ratios were standard-
ized against a male control sample and set arbitrarily at 1.0. Healthy female control samples with theoretical X chromosome gene dosage of 2 had a mean AR/BG of 1.84, approximately as expected. 

Four of the five tumor samples and their constitutional paired DNAs, when assayed in quintuplicate, showed AR/BG ratios that overlapped with those of the female controls. In two of these cases (tumors 1163 and 1380) the ratio in the tumor was significantly lower than that in the paired control, but was indistinguishable (at the 5% confidence level) from the female controls. This suggests that allele dosage at AR was not significantly reduced from diploidy in four of five tumors with LOH at AR, so the retained chromosomal segment/chromosome must have been duplicated either as part of or subsequent to the deletion event. In the fifth case (tumor 1085), the AR/BG ratio was significantly higher than the control, to a mean of 2.6 times the male controls and 1.4 times the female control. This tumor sample therefore showed increase of the AR gene dosage, probably to 3. Taken together with the observation of extensive LOH in this tumor, the most likely explanation is loss and replacement of an inactive chromosomal segment as described above and a further restricted duplication of a small segment containing AR. The key feature of these events at or near AR is thus preservation of a copy number of \( \frac{1}{H_{11350}} \) in the deleted regions accompanied by preservation of the activated methylation status of the duplicated/amplified alleles.

**DISCUSSION**

This study found X chromosomal deletions in 36% of metastatic cutaneous melanomas in females. This deletion frequency is consistent with previous estimates based on surveys of the cytogenetic literature (19), but because the X chromosome is functionally hemizygous, it indicates a much higher probability of complete loss or inactivation by the LOH event alone than would an equivalent rate on an autosome. On that basis, loci on X should be regarded as relatively frequent targets of deletion in melanoma and are likely to play a significant role in its tumorigenesis. The specific regions targeted by deletion show simi-
factor intersectin-1 (ARHGGEF9) between DXS1190 and DXS1194; and, finally, the AR between DXS1194 and DXS983.

In this Xq11–12 region, we have characterized the changes in copy number and parental chromosome associated with these deletion events to interpret the likely mechanisms of selection for them. The assay used (14) was previously validated in the context of copy number changes in other systems, successfully demonstrating integer differences in gene dosage. The tumors samples here contain variable amounts of stromal cells, and the tumor cells themselves potentially show different changes in copy number in different subpopulations. We obtained a level of signal/noise similar to that of Poropat et al. (14) in our modified version of their assay. Analysis of copy number at the ARTR locus indicated that the tumors with LOH show either no change or an increase, in one case, to three copies. The signal in most of the tumors was lower than an exact multiple of half the (diploid signal) value obtained from constitutional DNA at the same time. This suggests that these tumors were composed of a mixture of cells in which a minority had copy number reduction of ARTR.

The AR gene itself may confer a proliferative advantage to melanoma tumor cells in female patients and may be the target of selection in LOH events. However, it need not be the only candidate because cyclin B3 is also in the region affected. Malignant melanoma is generally considered to be a hormone-independent tumor, and clinical trials of endocrine treatment against estrogens, androgens, and progestins have shown little response in malignant melanoma (20). However, an increased mole count often observed during puberty and hypermelanization in pregnant women suggest that melanocytes may respond to steroid hormones, including androgens (21). In prostate cancer, tumors treated with antiandrogen therapy often show high-level amplification of AR gene copy number (7), and low level increases in AR gene copy number have been observed in untreated advanced tumors (22).

An 18.6-Mb proximal smallest region of overlap on the p arm may be resolved with a deletion frequency equal to that in the centromeric region between DXS999 and DXS1068. A number of genes in this region show potential for a role in tumorigenesis. These include PPEF1, a serine-threonine protein phosphatase with an EF-hand calcium-binding domain involved in sensory neuron differentiation; RUK, regulator of ubiquitin kinase; and PRDX4, a peroxidase with high activation of nuclear factor-κB protease.

A 6.2-Mb smallest region of overlap can be defined at Xp22 between the markers DXS8051 distally and DXS9902 proximally. Genes with possible involvement in cancer in this region include ocular albinism 1 (OA1), which regulates pigment production in skin and eyes (23), Ras-associated protein (RAB9), and epidermal growth factor-like 6 (EGFL6), although meningiomas are the only cancers known to express this gene.

Finally, a 23.9-Mb smallest region of overlap with a lower frequency of deletion (5 of 28 tumors, 18%) was found in distal Xq. This region is defined by the markers DXS1212 proximally and DXS1193 distally. Deletions in this region have been reported previously in prostate cancer (8), and a number of genes located in this area show potential for a role in cancer. SMARCA1 is a SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin structure. MTS4 is an Ste20-
related kinase that activates the MEK/extracellular signal-regulated kinase pathway, and FGF13 is a member of the fibroblast growth factor family expressed in the central nervous system.

These combined data clearly resolve four independent X chromosome smallest regions of overlap. Selective pressures should favor deletions that confer a growth advantage to tumor cell lineages. However, it may be going too far to suggest that all deletions in a tumor, such as tumor 1079 in this study, with three apparently independent regions of deletion, can necessarily be explained in terms of target genes alone. Some deletions arising through chromosomal instability, whether on the X chromosome or elsewhere in the genome, that may confer no growth advantage may be propagated by association with selectively advantageous regions in a growing tumor cell population.

Of particular interest was the observation that collectively, the more broadly defined smallest regions of overlap include most members of the MAGE gene family with the exception of the MAGE-A members on Xq28, a region distal to the study region. Most MAGE genes, with the exception of MAGE-D, are expressed exclusively in tumors and in the germ line (24). It is therefore possible that these deletions are selected during the tumorigenic process and lead to reduced MAGE gene expression with consequent reduction in immunogenic exposure of melanoma cells during tumor progression.

Knudson’s two-hit hypothesis (25) concerning the inactivation of TSGs states that both parental copies must be inactivated for a tumor cell to be formed or progressed. However, LOH (DNA deletion) events are seldom simply reduction to monosomy at the gene or marker concerned. In the study by de Nooij-van Dalen et al. (13), LOH events were mainly found to be due to somatic recombination or chromosome loss followed by duplication of the remaining chromosome. Other possible mechanisms include simple deletion of a chromosomal segment, nondisjunction (monosomy), loss of a chromosomal region with duplication from the remaining homologous chromosome (gene conversion), or translocation.

We found that every case with LOH at ARTR showed only a small residual signal after X inactivation analysis, in which the allelic ratio suggested random (polyclonal) X inactivation, presumably from contaminating adjacent stromal tissue. The fact that all samples showed the same pattern of LOH and X inactivation is evidence that LOH occurred via a similar mechanism in all these samples. This leads us to the conclusion that only one basic mechanism can explain these results: an inactive allele of the AR region was targeted by LOH and subsequently replaced by the remaining active allele. A mechanism involving tandem duplication may account for further gene amplification.

We found that the AR gene copies present in the tumor after the LOH event(s) were unmethylated and therefore active, at least doubling the number of active copies. These findings suggest that one of the selective pressures favoring the LOH event is, paradoxically, selection for increased activity of a dominantly acting putative melanoma oncogene. Could observed deletions target a TSG? The Knudson two-hit hypothesis seems incompatible with targeting of a chromosome that expresses few, if any, genes. Thus a further hypothesis could be that a putative TSG is expressed from the “inactive” chromosome at the stage of tumorigenesis at which these deletions occur. Furthermore, they may be preceded by breakdown of X inactivation brought about by prior oncogenic mutations elsewhere in the genome or in X itself. The absence of LOH events targeting the same putative TSG on the active chromosome could be explained in three ways. The first is that the initial preferred mode of activation of the TSG may be point mutation or that there may be a closely neighboring essential gene that leads to death of clones with deletions of the active chromosome in this region. Alternatively, the initial event may be an activating oncogenic point mutation on the active chromosome. We have shown that one

![Gene dosage assay results for melanoma samples showing LOH at ARTR. Gene copy number is calculated by normalization of male control ARBG (see “Materials and Methods”) PCR products ratios to 1 AR gene copy, and other results are scaled accordingly. Gene copy number is shown on the vertical axis, with tumor samples arrayed on the horizontal axis. Paired samples are shown (C, constitutional samples; T, tumor samples). Mean ARBG ratio values for quintuplicate replicate samples are shown with 95% confidence interval error bars. The extent of the 95% confidence interval range for all female control samples is shown by the horizontal lines. Four of five tumors (tumors 1380, 1069, 1163, and 1603) show no apparent gene dosage change, whereas one (tumor 1085) shows apparent gene amplification.](https://cincancerres.aacrjournals.org/content/6/7/6481/F4.large.jpg)
outcome of these events may be increased copy number of the active chromosome, with potential for increased dosage of dominantly acting oncogenes. Further mapping of these complex events and identification of the genes responsible will permit direct testing of these hypotheses.

REFERENCES
Frequent Loss of Heterozygosity Targeting the Inactive X Chromosome in Melanoma


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/9/17/6476

Cited articles
This article cites 24 articles, 5 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/9/17/6476.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/9/17/6476.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.