Frequent Loss of Heterozygosity Targeting the Inactive X Chromosome in Melanoma

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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 *DX1204* 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 *DXS981* 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*.

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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 *Hpa*II, 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 *Hpa*II 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 *Hpa*II 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).

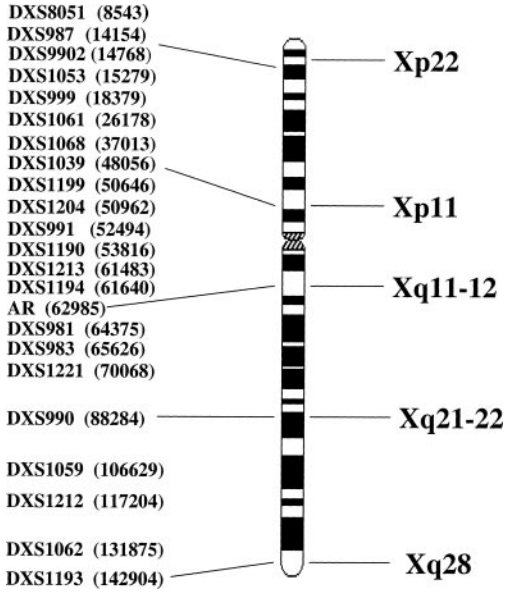

Fig. 1 Diagram of the X chromosome showing positions of microsatellite markers used in this study. Physical map distances are shown in *parentheses* after each marker. Cytogenetic positions, where available, are shown to the *right* of the drawing.

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.[3] 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-Scan 2000 DNA Analyzer (Corbett Research). 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 *Hpa*II (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 *Hpa*II-digested tissue DNA was predetermined to indicate that the remaining chromosome was in fact the active X.

[3] http://www.gdb.org.

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′-CTCTGCCTATTGGTCTATTTTCCC-3′, and the reverse primer used was 5′-GAAAACATCAAGGGTCCCATAGAC-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 $MgCl_2$ 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 nondenaturing 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× 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/$\sqrt{n}$). 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 *DXS1204* 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 *DXS8051* 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 reduction compared with blood DNA. Samples of *Hpa*II-digested tumor DNA showed reduced *ARTR* PCR product yield 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-

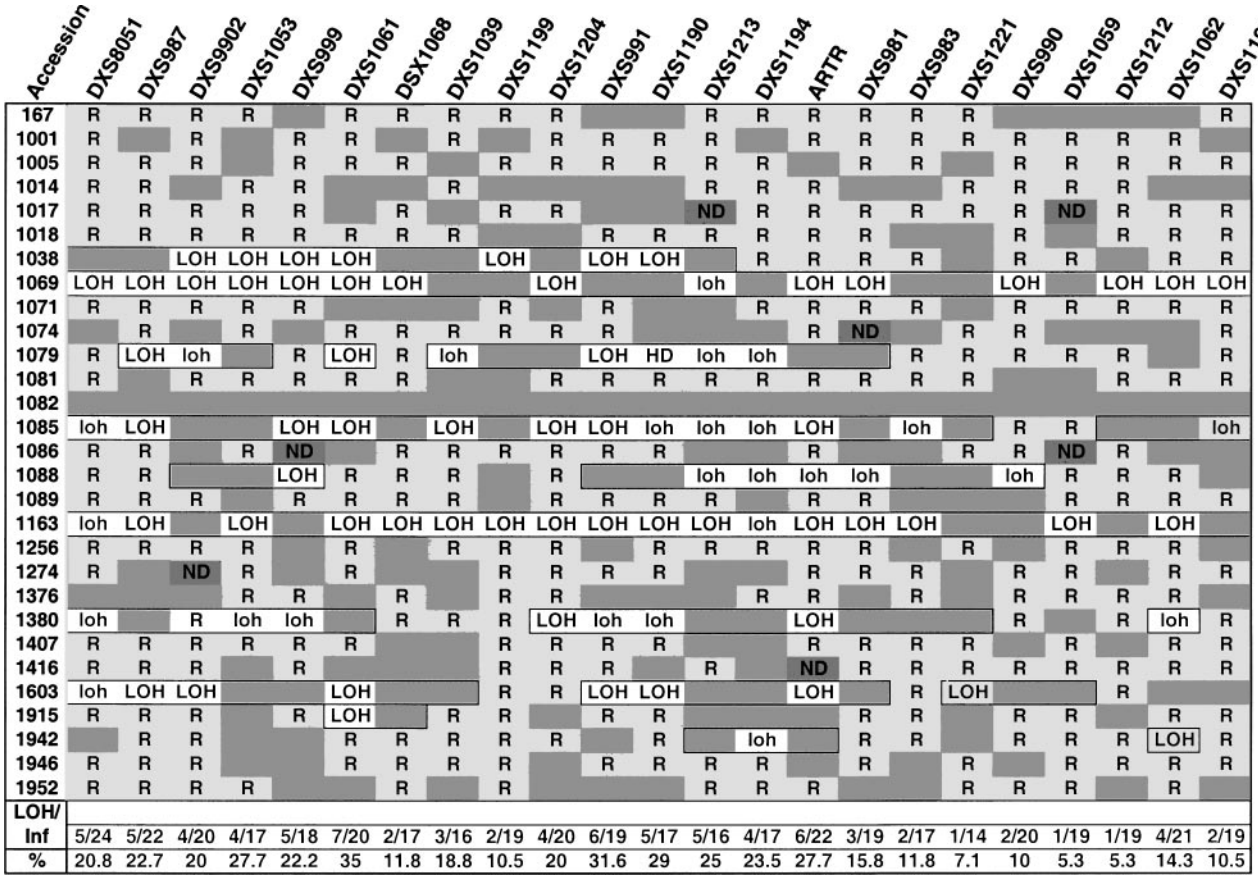

Fig. 2 Deletion map of the X chromosome in female metastatic melanoma patients. Patients, identified by accession number, are presented in rows, and microsatellite markers are arranged in proximal to distal order. Cells marked *R* show heterozygosity with both alleles retained, empty cells shaded *gray* represent homozygosity (uninformative), and cells marked *LOH* or *loh* represent loss of heterozygosity (LOH). Samples showing LOH with more than 50% allelic shift are in *uppercase*, and samples showing LOH with allelic change between 30% and 50% are in *lowercase*. *Dark gray cells* marked *ND* show results not determined. Tumor 1079 shows a putative homozygous deletion, marked *HD* at marker *DXS1190*. Tallies for samples with LOH calculated as a percentage of total informative samples are shown at the *bottom* of the figure. The androgen receptor exon 1 trinucleotide CAG repeat is abbreviated *ARTR*.

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 ≥ 2 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-

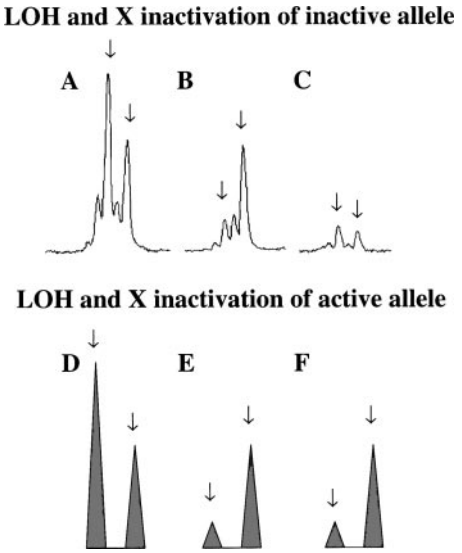

Fig. 3 Microsatellite profiles obtained at the *ARTR* locus using the Corbett Gel-Scan DNA Analyzer. The *vertical axis* corresponds to signal voltage, and the *horizontal axis* is gel retention time. Microsatellite alleles are marked with *arrows*. *A*−*C* show results for melanoma patient 1163. *A*, constitutional profile obtained from peripheral blood leukocyte sample. *B*, tumor sample showing loss of heterozygosity (LOH) with reduction of the smaller allele. *C*, profile obtained after X inactivation analysis showing reduction of the larger remaining unmethylated and active allele. Remaining signal is residuum from stromal contaminant DNA and shows random (polyclonal) X inactivation status. *D*−*F*, comparative diagram illustrating expected results for LOH targeting the active allele. *D*, constitutional profile. *E*, tumor profile showing reduction of the smaller allele. *F*, profile showing expected result after X inactivation analysis. In this case, where the active tumor allele is already targeted by LOH, further allelic reduction is not possible, and little further change is seen.

larities to those observed in a previous study of breast cancer (4), suggesting some common underlying genetic features.

The Xq11–12 region containing the *AR* gene is frequently amplified or deleted in a number of cancer types (5–7), and this has also been found to be true in this study of female melanoma patients. In this case, LOH analysis has defined a 14.9-Mb region straddling the centromere, between the microsatellites *DXS1204* and *DXS983*. Two tumors with restricted deletions (tumors 1088 and 1603) narrow the p arm region to *DXS1204* with a q arm boundary at *DXS983*, and this region is supported by retention of heterozygosity in two tumors (tumors 1079 and 1603). Several other tumors suggest the region can be further narrowed, perhaps as far as the *DXS1190* to *DXS981* interval defined by tumor 1942. The importance of this interval is highlighted by a probable homozygous deletion of *DXS1190*. The *DXS1204* to *DXS983* region is relatively gene poor and of course includes the centromeric gap in which completeness of the physical map and genome sequence cannot be assumed. For reasons explained below, TSGs and/or oncogenes may have a role to play with the following known genes of particular relevance: cyclin B3 (*CCNB3*) and the melanoma antigen *MAGED2* between *DXS1039* and *DXS1991*; a ras-like GTPase (*RAGB*) and ubiquitin ligase (*UBQLN2*) between *DXS991* and *DXS1190*; the Cdc42-interacting guanine nucleotide exchange factor intersectin-1 (*ARHGEF9*) 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 the germ line, successfully demonstrating integer differences in gene dosage. The tumor 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-

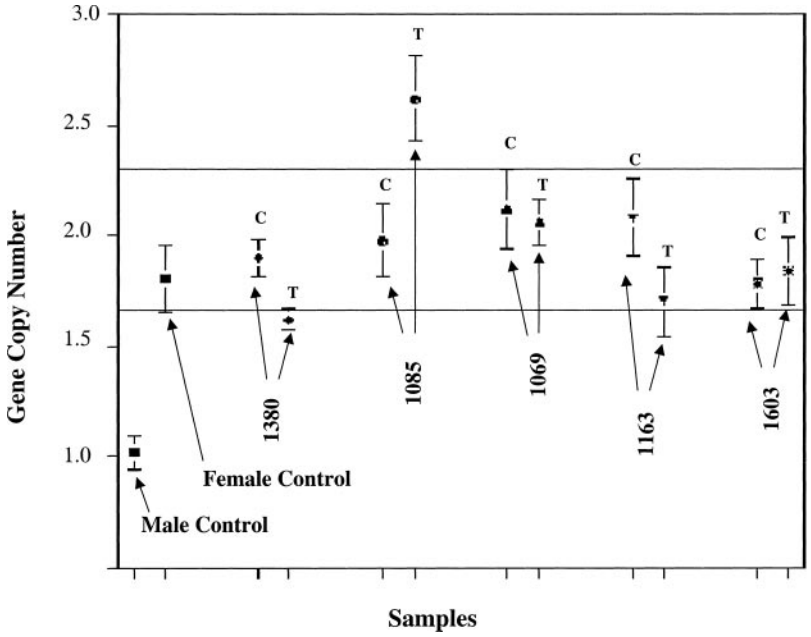

Fig. 4 Gene dosage assay results for melanoma samples showing LOH at *ARTR*. Gene copy number is calculated by normalization of male control *AR*/*BG* (see "Materials and Methods") PCR product ratios to 1 *AR* gene copy, and other results are scaled accordingly. Gene copy number is shown on the *vertical axis*, with tumor samples arrayed on the *horizontal axis*. Paired samples are shown (*C*, constitutional samples; *T*, tumor samples). Mean *AR*/*BG* 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.

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 progress. 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

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.

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