Tumor and Stem Cell Biology

Chromosome 10, Frequently Lost in Human Melanoma, Encodes Multiple Tumor-Suppressive Functions *

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Abstract

Although many DNA aberrations in melanoma have been well characterized, including focal amplification and deletions of oncogenes and tumor suppressors, broad regions of chromosomal gain and loss are less well understood. One possibility is that these broad events are a consequence of collateral damage from targeting single loci. Another possibility is that the loss of large regions permits the simultaneous repression of multiple tumor suppressors by broadly decreasing the resident gene dosage and expression. Here, we test this hypothesis in a targeted fashion using RNA interference to suppress multiple candidate residents in broad regions of loss. We find that loss of chromosome regions 6q, 10, and 11q21-ter is correlated with broadly decreased expression of most resident genes and that multiple resident genes impacted by broad regional loss of chromosome 10 are tumor suppressors capable of affecting tumor growth and/or invasion. We also provide additional functional support for Abilim1 as a novel tumor suppressor. Our results support the hypothesis that multiple cancer genes are targeted by regional chromosome copy number aberrations. Cancer Res; 74(6); 1814–21. ©2014 AACR.

Introduction

Increasingly high-resolution genomic studies have established that recurrent focal deletions and amplifications in cancer can selectively target specific oncogenes and tumor suppressors (1, 2). In melanoma, MITF, CCND1, BRAF, CDKN2A, and PTEN are some of the validated oncogenes and tumor suppressors targeted by such focal copy number-changing aberrations (3–5). However, there are also many recurrent large regional or arm-level losses and gains that affect multiple resident genes (6, 7). One hypothesis states that most of these represent bystander passengers deleted or gained coincidentally along with the driver genes. However, increasing evidence is pointing toward the presence of multiple cancer driver genes targeted by these regional alterations. Recent papers have described multiple tumor growth suppressors in broad regions of loss, in an in vitro genome-wide assay (8) and in a targeted in vivo screen of chromosome 8 for liver cancer (9).

In melanoma, loss of the entire chromosome 10 was identified as early as 1991 (10). On the basis of focal deletions at 10q23.3 and the presence of loss-of-function mutations, PTEN was functionally validated as the genetic target of the loss (11, 12). However, focal inactivation of PTEN is seen in only approximately 10% of melanomas, whereas copy number loss of the entire chromosome 10 is observed in >50% of melanomas (7). In contrast, the observed approximately 60% of CDKN2A/B deletions on chromosome 9p are focal. These data suggest that although PTEN is a major driver of chromosome 10 loss, other genes may also be targeted for inactivation. Here, we ask whether recurrent losses on human chromosomes 6, 10, and 11 in melanoma may broadly target multiple tumor suppressors acting at different steps in tumor progression.

Materials and Methods

Determination of the 48 candidate genes

We previously published microarray expression data containing nine nevi, 25 primary melanomas, and 63 metastatic melanomas (6), which are now available publically on the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE46517. We first generated a list of 144 genes that are significantly downregulated in the metastases compared with either the primary melanomas or nevi, based on the following criteria: P < 0.05, Student t test; >1.3-fold average decrease in expression; and >50% present calls in the primary melanoma or nevi group (13). We then intersected this list with an identical analysis of the data from Talantov and colleagues (14) and Riker and colleagues (15). On the basis of the publicly available probe data at the time of analysis, a total of 62 annotated genes were in common between our data and at least one of the other lists. Five genes were removed because of poor or no homology between human and mouse (AKR1C1, AKR1C2, AKR1C3, C10orf116, and P53MIP1). Of the remaining 57 genes, pLKO-based short hairpin RNAs (shRNA) were available for 48 (Supplementary Fig. S2).

Cell culture

The establishment and maintenance of inRAS cell lines have been previously described (16). MUM2C, WM115, SKMel28, and 1205Lu human cell lines were maintained as mycoplasma-free

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cultures in RPMI, 10% FBS. The MMM-7.1 primary melanocyte culture was initially isolated from 2-day-old FVB.CDKN2A−/− pups as previously described (17). Low-passage cultures were simultaneously retrovirally infected with a pBabe-hygro-RRAp1V600E and a pRetroSuper-shPten construct (18) and selected with hygromycin and puromycin. MMM-7.1 cultures were subsequently maintained in RPMI, 10% FBS, 200 nm 12-tetradecanoylphorbol 13-aceta (Sigma), and 200 pm cholera toxin (Sigma).

Mouse studies
Eight iNRAS cell lines were tested for in vivo tumor formation by intradermal injection of 5 × 10⁵ cells into the flanks of nude mice (Taconic). All iNRAS cell lines require doxycycline to express oncogenic NRAS. All mice were therefore maintained on doxycycline chow (Harlan–Teklad). Two cell lines, iNRAS-463 and iNRAS-485, were selected for further study on their relatively long tumor latencies. For human xenografts, 1 × 10⁶ cells were injected intradermally into the flanks of nude mice. For shRNA studies using the pTRIPZ-inducible vector, mice were maintained on doxycycline chow. For experimental lung metastasis assays, 5 × 10⁵ B16F10 cells were injected into the tail vein of nude mice. Mice were sacrificed 15 days later and lung nodules were counted under a dissecting microscope. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees at the Dana-Farber Cancer Institute (Boston, MA) and the University of Texas MD Anderson Cancer Center (Houston, TX).

In vivo shRNA screen
pLKO plasmid constructs targeting 48 genes were obtained as a generous gift from from Dr. William Hahn (Dana-Farber Cancer Center, Boston, MA). Four to five shRNAs were extracted and pooled per gene from equally mixed portions of bacteria using a Midiprep Kit (Qiagen). These were then further equally pooled according to chromosomal location (see Supplementary Fig. S2) before viral packaging and infection of iNRAS cell lines. All cells were transduced at a multiplicity of infection (MOI) of 0.5 without drug selection. After one day of recovery from the viral infection, cells from each pool were injected intradermally into both flanks of nude mice, using 5 × 10⁵ cells per injection. Mice were monitored for tumor formation and tumor sizes were measured every 2 to 3 days. For secondary shRNA validation, individual or pooled shRNAs were packaged and transduced into the target iNRAS cells. Transduced cells were selected using puromycin (Sigma) for 2 days, then expanded for one passage, and injected into nude mice as described above. Real-time reverse transcription PCR (RT-PCR) for mouse Tacc2, Tcf7l2, and Ablim1 used the following primers: mTacc2-F: CCT TTG AGA CCC CCG AGT, mTacc2-R: AAC ACC GCC GAG GAG GAG, mTcf7l2-F: CCC ACC ATG TCC ACC CAC, mTcf7l2-R: ATT TGT CCT AGC GTG CCC, mAblim1-F: ATT TAG CAG CCA TCC CCA, mAblim1-R: CCA TCC CGG ACA TCT TGA.

Sequencing of shRNAs from the in vivo screen
Tumors were necropsied from mice when they reached 2 cm in diameter or when the mice became moribund. The tumors were removed of normal skin and then flash frozen in liquid nitrogen. To sequence the shRNAs, two portions from opposite sides of each tumor were sampled. DNA was isolated using the DNeasy Kit (Qiagen) and PCR was carried out using pLKO-specific primers that flanked the hairpin: pLKO-seq-F, GAG GCC CTA TTT CCC ATG and pLKO-seq-R, GAT CTC TGC TGT CCC TGT A. PCR samples were run on agarose gels and visually confirmed to produce a single 490 bp band. Standard Sanger sequencing was then performed, with each tumor piece undergoing both forward and reverse sequencing. As a control, an shGFP tumor was also sequenced and confirmed.

In vitro invasion
Using the per-gene pooled four to five shRNAs isolated for the in vivo screen, cells from the iNRAS-463, iNRAS-650, and MMM-7.1 lines were infected in 24-well plates, one gene per well. An MOI of 2 was used to ensure 100% infection without drug selection. After 2 days of recovery from viral infection, cells were trypsinized for use in the invasion screen. The screen was carried out using 96-well Matrigel-coated invasion chambers (BD Biosciences) according to the manufacturer’s instructions. Briefly, 1 × 10⁴ cells per gene were plated per well, in triplicate, in serum-free RPMI and allowed to invade toward RPMI containing 10% FBS, over a period of 20 to 22 hours. Invaded cells were fixed and stained with Calcein AM, then quantified using a fluorescent plate reader. Simultaneously, an equal number of cells was seeded into cell culture wells containing RPMI 1% FBS as loading controls. Invasion results were normalized against these loading controls. High-scoring genes were validated in 24-well invasion chambers (BD Biosciences), using 5 × 10⁴ cells per well.

Human Ablim1 plasmids
A lentiviral-inducible shRNA against human Ablim1 in the pTRIPZ vector was purchased (Open Biosciences). Knockdown was confirmed by RT-PCR using the following primers: hAblim1-F, AAT GAG AAT GGA CCG AGG AG and hAblim1-R, CCA AAG ATT TCC CGA AAC AC. An Ablim1 overexpression construct was generated using Gateway cloning technology (Invitrogen) according to manufacturer’s instructions. Briefly, a pENTR construct containing a full-length Ablim1 ORF (Origene, identical to NM_001003407) was cloned into a Gateway-compatible phAGE vector (19) using LR Clonase II.

Results
Loss of large chromosomal regions is associated with decreased gene expression
We hypothesized that large regions of chromosomal loss might affect multiple tumor suppressor genes in melanoma. To narrow down regions of interest, we reanalyzed our previously published DNA copy number data from patient samples (6). Genomic nonnegative matrix factorization analysis had stratified the samples into three groups, of which the k1 and k2 populations had a significantly worse survival than k3 (6). We noted a strong enrichment in the k1 and k2 populations for broad regional losses of the 6q, 10, and 1q21-ter chromosomal regions (Supplementary Fig. S1). These three regions are also
significantly lost in several other published melanoma datasets (7, 20, 21), including one in which poor survival was significantly correlated with loss of these three regions, among others (20). On the basis of our hypothesis, we focused on these regions as likely to be enriched for tumor suppressors.

We asked whether the loss of these regions perturbed the expression of the resident genes. We performed a cross-platform comparison of metastatic samples using matched DNA copy number and RNA expression data from The Cancer Genome Atlas (TCGA; Research Network, https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm, DOI # 2012-10-04). For each of the three regions, relevant samples were grouped into showing either chromosome loss (\(< -0.5\), log2 value) or retention of two copies (\(> 0.2\), \(< 0.2\)). We identified a strong association between decreased copy number and region-wide decreased gene expression (Fig. 1). This is consistent with our hypothesis that broad DNA copy number losses can lead to the decreased gene expression of multiple putative tumor suppressors. To focus on a set of genes likely to have a functional tumor-suppressive role, we defined a set of 48 genes that are significantly downregulated in metastatic melanomas compared with either primary melanomas or benign nevi, by intersecting our previous data (6) with two other published microarray datasets (Supplementary Table S1 and Supplementary Fig. S2; refs. 14, 15).

**Multiple genes on chromosome 10 are in vivo tumor growth suppressors**

We next screened this list of 48 candidate tumor suppressors for *in vivo* tumor suppression activity via RNA interference (RNAi). First, we sought a well-characterized model system for screening these genes for tumor suppressor activity. We have previously described the iNRAS mouse model of melanoma (16), from which we have characterized multiple primary tumor-derived cell lines for their *in vivo* allograft growth. Two lines, iNRAS-485 and iNRAS-463, were selected to serve as the system for this functional genomic screen based on their relatively long latency (5–8 weeks). Four to five lentiviral shRNAs targeting each of the 48 candidate genes were pooled by genomic location. Cells were virally transduced at an MOI of 0.5 and injected into nude mice without any drug selection, to allow shRNA-bearing cells to compete with each other and with nontransduced cells.

In both of the iNRAS cell lines, tumors harboring shRNAs against genes in the 6q and 10, but not the 11q21-ter pools, exhibited increased growth rates compared with both a non-targeting shRNA control (shGFP) and the parental lines (Fig. 2A and B and Supplementary Fig. S3). In the iNRAS-463 cohort, chromosome 10 shRNAs were split into three equal groups based on chromosomal location (Supplementary Table S1). Only one of these three groups showed increased growth rate, group 10-3 (Fig. 2B).

If shRNA-mediated gene downregulation drives the observed increase in growth rates, we reasoned that these oncogenic shRNAs would be enriched in the individual resultant tumors. To identify such shRNAs, tumors exhibiting significantly increased growth in the iNRAS-485 line were sequenced by the Sanger-based method. In 24 of 36 tumors with high-quality sequencing reads, a single shRNA was identified (Fig. 2C), suggesting that such an shRNA was positively selected for driving the enhanced tumor growth. Importantly, all shRNAs were identified from their assigned pool, confirming that no cross-contamination had taken place. Of the 24 identified shRNAs, two genes were represented seven times.

![Figure 1. Genomic loss (red boxes, top) correlates with broadly decreased gene expression (red boxes, bottom) in the chromosome 6q, 10, and 11q21-ter regions. Each column is data from one melanoma, taken from TCGA. The color scale represents log2 data: for DNA, it indicates copy number and for RNA, it indicates median centering of expression levels across all samples. Only samples that showed either loss (\(< -0.5\)) or retention of two copies (\(> -0.2\), \(< 0.2\)) in each region are displayed (n = 135, 140, 150, respectively).](image-url)
(enriched in seven independent tumors) each: Tacc2 and Tcf7l2 (Fig. 2C), both of which reside on chromosome 10.

We next performed secondary validation on all of the candidate tumor suppressors. First, to initially validate Tacc2 and Tcf7l2, we pooled the five shRNAs per gene and generated stably knocked down cells. These cells formed tumors at significantly accelerated rates compared with controls (Fig. 3A). Other identified single shRNAs targeted the following genes: Ablim1, Net1, Lama2, Pdlim1, Tpd52l1, and Perp (Fig. 2C). The individual shRNAs sequenced from the initial screen were tested singly. Of these genes, only shRNAs targeting Ablim1 and Net1 significantly increased the tumor growth rate (Fig. 3B).

For tertiary validation, we selected our highest performing genes, Tacc2, Tcf7l2, and Ablim1 for the testing of multiple single hairpins each to rule out off-target effects. For both Tacc2 and Tcf7l2, we identified two shRNAs with strong knockdown efficiency, including the shRNAs identified by sequencing (shTacc2 #4 and shTcf7l2 #4). For both genes, these shRNAs each significantly enhanced the growth rate of the tumors (Fig. 4A and B). For Ablim1, we used all five available shRNAs because four of the five gave strong knockdown. Of these, three significantly decreased tumor latency in a third cell line, iNRAS-413 (Fig. 4C), and all three scoring hairpins knocked down Ablim1 to a similar degree.

To address whether these colocalized tumor suppressors on chromosome 10 function cooperatively, we assessed the potential of Tacc2 and Tcf7l2 in combination and discovered that they did not significantly cooperate in affecting tumor latency or growth (Supplementary Fig. S3). These data suggest that the loss of these two genes may be phenotypically redundant or functionally parallel. Supporting these results is the observation that Tacc2, Tcf7l2, and Ablim1 were all single-scoring genes within the 10-3 pool in the iNRAS-463 in vivo tumorigenesis screen (Fig. 2B and Supplementary Table S1).

Multiple genes on chromosome 10 are in vitro tumor invasion suppressors

As shown above, our in vivo functional genetic screen identified three suppressors of in vivo tumor growth, all
knocked down, enhanced invasion in at least two of the three cell lines, including four that scored in all three (Fig. 5B). The top hit, Acta2, has been identified in a published in vitro shRNA invasion screen (22), supporting the validity of our results. Secondary validation was performed in 24-well Boyden chambers, confirming the anti-invasion effects of five of the eight candidate suppressors: Ablim1, Acta2, C10orf57, Adra2a, and Ank3 (Fig. 5C), all of which are located on chromosome 10.

**Functional analysis of Ablim1**

As Ablim1 was the only gene to score highly in both in vivo tumorigenesis and in vitro invasion assays, we selected it for further validation using human cell systems. First, we confirmed that Ablim1 mRNA levels correlate significantly with the copy number status of chromosome 10 (Fig. 6A) in the TCGA patient dataset, consistent with it being one of the genes targeted by broad chromosomal loss. Next, using human cell lines selected for their baseline phenotypes (Supplementary Table S2), we showed that Ablim1 knockdown resulted in a protumorigenesis phenotype manifested as a consistent, though modest, increase in tumor penetrance in two independent human cell lines, WM115 ($P = 0.1$) and 1205Lu ($P = 0.02$), by Kaplan–Meier analysis (Supplementary Fig. S4), although the rate of growth in vivo or in vitro was not enhanced (Supplementary Fig. S4). On the other hand, knockdown of Ablim1 significantly enhanced the invasion of the poorly metastatic human WM115 cell line in vitro (Fig. 6B), whereas reciprocally, Ablim1 overexpression decreased invasion in the human SKMel28 and MUM2C cell lines compared with GFP (Fig. 6C). We next asked whether Ablim1 can suppress invasion in vivo, in an experimental metastasis model. Indeed, overexpression of human Ablim1 in the highly metastatic mouse cell line B16F10 resulted in a significant reduction in lung nodules after tail vein injection (Fig. 6D; $P = 5 \times 10^{-4}$). These data collectively validate Ablim1 as a novel tumor suppressor in melanoma.

**Discussion**

In cancer, recurrent losses and gains of broad chromosome regions suggest that multiple genes in the same region may be cotargeted along with established tumor suppressors and oncogenes. Here, we provided functional evidence that in melanoma, recurrent loss of the entire chromosome 10 results in the repression of multiple tumor suppressors collectively regulating more than one step of cancer. A targeted RNAi screen of 48 genes, selected on the basis of expression levels in progressed human melanomas, revealed that knockdown of Tacc2, Tcf7l2, and Ablim1 enhances tumor growth in vivo, whereas knockdown of Ablim1, Acta2, and three other genes enhances invasion in vitro.

Consistent with our results, a recent study in melanoma also comparing DNA and RNA platforms identified 6q and 10q loss as significantly associated with ulceration, a negative prognostic indicator (21). Interestingly, their in silico analysis identified Ablim1 as the only gene on 10q with a significant DNA and RNA correlation. We also note that all of our validated hits lie on 10q. Nevertheless, as our screen was purposefully targeted, it is likely that other genes resident on chromosome 10 may also be melanoma suppressors. Some
possibilities include CUL2 and KLF6, which have been proposed as candidate targets in melanoma based on in silico analyses (7). Consistent with published data, all four of our top hits have previously been described as tumor suppressors. Tcf7l2 was a top hit in an in vitro shRNA screen in colon cancer (23) and has
been validated as an in vivo tumor suppressor using a mouse knockout model (24). Tacc2 was initially described as a breast tumor suppressor (25), and has since been described as either a tumor suppressor or oncogene (26, 27), depending on the cell type context. Acta2 was a top hit in another melanoma RNAi screen, where knockdown strongly enhanced invasion through a three-dimensional collagen/Matrigel plug (22). Finally, Ablim1 was a significant hit in an in vivo retrotransposon mutagenesis screen, where multiple inactivating insertions were documented in tumors of p19- and p53-deficient mice (28). Interestingly, reanalysis of published iNRAS mouse microarray data (16) found that NRAS signaling represses the expression of Ablim1, Acta2, and Tcf7l2 (Supplementary Fig. S5). Furthermore, in the TCGA metastatic sample dataset, samples with BRAF or NRAS hotspot mutations, but not NF1 mutations, were significantly enriched for chromosome 10 loss (Supplementary Fig. S5). These in silico findings suggest that chromosome 10 loss and canonical mitogen-activated protein kinase signaling may corepress these tumor suppressors.

Although we validated multiple hits on chromosome 10, shRNAs on chromosome 6 (Lama2, Perp, Tpd52l1, Slc45a2) failed to validate as single shRNAs in our secondary screens. One possibility is that these shRNAs were false positives. Another possibility is that chromosome 6 shRNAs require cooperation to affect the phenotype. Indeed, the results of our shRNA sequencing show that 63% (10/16) of the chromosome 6 tumors harbored multiple shRNAs, in contrast with zero of 15 of the chromosome 10 tumors (Fig. 2C). This suggests that, unlike chromosome 10, the majority of chromosome 6 tumors may have required multiple coexisting shRNAs to enhance tumor growth. Further study will be required to deconvolute these shRNA pools and to test their combinations. Finally, none of the chromosome 11 shRNAs scored in either of our primary screens, suggesting that other genes in the region may be the true targets of the recurrent loss.

In summary, our study demonstrates that loss of chromosome 10 in melanoma likely targets more than just the PTEN tumor suppressor and that these other genes may collectively or redundantly act at more than one step in progression. This provides functional data in support of the thesis that broad regional loss of chromosomes is one mechanism to target multiple tumor suppressors in cancers.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: L.N. Kwong, L. Chin
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