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Award Number: W81XWH-08-1-0584

TITLE: Dissecting the Molecular Mechanism of RhoC GTPase Expression in the Normal and Malignant Breast

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REPORT DATE: September 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-09-2009		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 09/01/08 - 08/31/09
4. TITLE AND SUBTITLE Dissecting the Molecular Mechanism of RhoC GTPase Expression In the Normal and Malignant Breast			5a. CONTRACT NUMBER	
			5b. GRANT NUMBER W81XWH-08-1-0584	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) John Brenner Email: chadbren@med.umich.edu			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Michigan Ann Arbor, Michigan 48109-1274			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)	
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT enter a brief (approximately 200 words) unclassified summary of the most significant finding during the research period. Primary inflammatory breast cancer (IBC) accounts for approximately 3% of new breast cancers in the US. This form of locally advanced breast cancer is characterized clinically by erythema, warmth, and dimpling of the skin that arise rapidly. In fact, IBC is not associated with precursor lesions, but <u>is rapidly invasive</u> , highly angiogenic and metastatic <u>from onset</u> . IBC has previously been characterized by the Merajver lab to have increased RhoC expression as compared to stage matched controls. Despite this the molecular mechanisms giving rise to the disease, and to RhoC overexpression, have not been characterized. In this project, we have postulated several potential molecular mechanisms that may lead to RhoC overexpression. Thus far, we have developed a method for gene fusion identification from paired end sequencing data, and used SOLEXA high throughput massively parallel sequencing to identify potential gene fusion candidates in two IBC cell lines. Likewise, we have also completed aCGH and microRNA profiling analysis of these cell lines. Unexpectedly, we found that two IBC cell lines both harbor an extra copy of Chromosome1 and are currently studying the significance of this association.				
15. SUBJECT TERMS No subject terms provided.				
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 49
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U		
				19b. TELEPHONE NUMBER (include area code)

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Introduction:

Primary inflammatory breast cancer (IBC) accounts for approximately 3% of new breast cancers in the US. This form of locally advanced breast cancer is characterized clinically by erythema, warmth, and dimpling of the skin that arise rapidly, typically within six months. IBC is generally not associated with precursor lesions and is rapidly invasive from the outset, especially to the skin and lymphatics, and is highly angiogenic and metastatic. Because of this disease's rapid progression, the effectiveness of aggressive multimodality treatment is limited; the 5-year disease-free, mean survival rate is less than 45%, making IBC the most lethal form of breast cancer (1). This rapid progression is due to the development of distant metastases, indicating that the tumors quickly acquire the ability to invade and metastasize during tumor development. This suggests that the unique aggressive inflammatory phenotype of IBC is the result of a limited number of concordant genetic alterations. As such, IBC constitutes an excellent paradigm to understand aggressive phenotypes in breast cancer. Previously, our laboratory has found concordant and consistent overexpression and of RhoC GTPase in tissue samples from patients with IBC as compared to stage-matched non-IBC (2, 3). We have also demonstrated that RhoC GTPase occupies an integral role in the aggressive phenotype of IBC (4, 5). With the increasing evidence that RhoC and other ras-homology family proteins play a significant role in other cancers (6, 7), the therapeutic importance of inhibiting RhoC activity is clear, highlighting the crucial need to uncover the molecular mechanisms leading to RhoC-driven metastatic phenotype of IBC. In spite of this need, however, a model explaining the mechanisms of RhoC overexpression in breast cancer does not exist. The goal of this award is to establish such a model. ***Our central hypothesis was that overexpression of RhoC GTPase in metastatic breast cancer is due to gene amplification, epigenetic deregulation, transcription factor deregulation, and/or enhanced or differential mRNA stability. Because of these cellular and molecular alterations, early stage IBC is subject to rapid metastatic spread through downstream effectors signaling for invasion and angiogenesis.***

Body:

As I matriculated through graduate school, I originally thought that I was going to work full time in Dr. Merajver's lab (first graduate school rotation) studying IBC. However, as I was granted this award I was also choosing to transfer into Dr. Arul Chinnaiyan's lab. Fortunately, I was granted permission by the DOD to transfer the award to follow me to continue working on this project from Dr. Chinnaiyan's lab. Because of this, we have been able to establish an effective and highly collaborative meeting with Dr. Merajver where I attend her bi-weekly lab meetings and work with a technician in her lab to help complete this project. This has given me a lot of unique experiences. For example, learning how to create well defined experimental protocols and making sure that she has the appropriate materials and controls to execute each experiment. As such, continuing this DOD pre-doctoral grant has given me the opportunity to continue existing collaborations and to continue improving my leadership skills through working with a technician on a daily basis.

In addition to working with a technician, I have also had the opportunity to train three undergraduate students through the University of Michigan Undergraduate Research Opportunities Program and one Master's degree student. The students have learned several different protocols including PCR, restriction digests, Gateway cloning, DNA miniprep, DNA maxiprep, RNA isolation, cDNA synthesis, qRT-PCR, PCR, Western blotting, transfections of both large DNA vectors and siRNA into mammalian cells, cell culture, production of lentivirus and lentiviral transduction, cell invasion assays, cell growth assays and propidium iodide staining. Additionally, I have led a bi-weekly cancer biology journal club meeting with all of the students in our lab (22 undergraduates). At the end of each semester, I help the students

compile their results to present at a lab meeting and at an Undergraduate research forum by both poster presentation and lecture. Importantly, one of the students that I have been training was awarded an NIH summer fellowship that funded her to work in the lab for the entire summer.

While I have found the opportunity to teach students standard techniques on a daily basis and have found reward in the successes that they have experienced, I have also been able to learn several new experimental techniques that I would not otherwise have had the opportunity to learn without this training grant including Solexa high throughput Transcriptome sequencing, Fluorescence in situ hybridization as well as running aCGH and microRNA arrays. Perhaps more interesting is the analysis algorithms that I am helping to develop, including those used to identify novel gene fusions from paired end sequencing data (8), for the analysis of my global profiling data from these IBC cell line samples. While little is known about the molecular origins of inflammatory breast cancer, we have made significant advances not only in the acquisition of large profiling data sets of DNA copy number, microRNA expression and transcriptome sequencing, but also in software development to analyze this data. Currently, we are in the process of completing an integrated analysis from all three profiling platforms. Additionally, we have unexpectedly found that the two IBC cell lines SUM149 and SUM190 have an extra copy of chromosome 1. Because several other stage matched breast cancer cell lines do not have this extra copy of chromosome 1, we are exploring the occurrence of chromosome 1 amplification in IBC clinical samples. The significance of this finding is still unclear, but will be explored in more detail if a clinical correlation is observed.

The opportunity to work on developing novel techniques and protocols for this project has led directly to opportunities to improve my communication and professional skills. Within the last year, I have presented some of the work at the American Association for Cancer Research Meeting in Denver, Colorado (April 2009). At that meeting, I was a co-author on three posters on both the role of RhoC GTPases in IBC and other breast cancers as well as co-author on an abstract presented by podium presentation. Additionally, I received a scholarship to attend a keystone conference in Victoria, British Columbia and received a nomination to become an American Association of Cancer Research Associate council member.

Key Research Accomplishments:

Specific Aim 1: *To delineate if and how gene amplification in RhoC GTPase occurs in breast cancer and to identify novel gene fusions in inflammatory breast cancer.*

- Completed RhoC FISH and discovered that IBC cell lines do not have amplification of the RhoC locus, but carry an extra copy of chromosome 1.
- Acquired 244k Agilent aCGH data for several cell lines including the two IBC cell lines, SUM149 and SUM190.
- Completed the Illumina bead station microRNA profiling chip V2 of cell line panel including HME, MCF10A, SUM149, SUM190, MDA-MB-231, HCC1937 and BT20.
- Sequenced the RNA transcriptome of both SUM149 and SUM190 using massively parallel, high throughput paired-end sequencing on a SOLEXA GA2 from Illumina.

Specific Aim2: *To determine how DNA methylation status and histone modifications regulate the RhoC GTPase promoter, and to assess the ability of the small molecule drugs 5-azacytidine and Trichostatin A to alter the metastatic phenotype depicted by an IBC cell line model.*

- Completed Illumina bead station microRNA profiling chip V2 of cell line panel including HME, MCF10A, SUM149, SUM190, MDA-MB-231, HCC1937 and BT20 treated with 5-azacytidine or Trichostatin A.
- Prepared RNA transcriptome libraries of both SUM149 and SUM190 treated 5-azacytidine or Trichostatin A for sequencing on an Illumina SOLEXA GA2.
- Treatment of MCF10A and HME cells with either 5-azacytidine or Trichostatin A revealed no significant increase in RhoC mRNA expression suggesting that the molecular mechanism leading to RhoC overexpression does not involve the activation of genes repressed by either methylation or deacetylation.

Specific Aim3: To characterize the consequences of down regulating the expression of the transcription factors FoxP3, HoxA3, HoxB7, HoxB8, HoxD9, HoxD10, CREB and NFκB1, all of which contain highly conserved binding sites in the putative RhoC GTPase promoter, on molecular pathways regulating cell proliferation, survival and the metastatic phenotype, using an RNAi model system of human IBC cell lines.

- Established stable shRNA knockdown cell lines for FoxP3, HoxA3, HoxB7, HoxB8, HoxD9, HoxD10, CREB and NFκB1 in SUM149 cells.
- Identified NFκB1 as a key regulator of RhoC mRNA and protein expression in SUM149 and SUM190 cells.
- Established a 4.0kbp RhoC promoter reporter system.
- Developed site mutants of RhoC promoter reporter system.
- Completed chromatin immunoprecipitation assays that demonstrated enhanced NFκB1 binding at 2/3 putative NFκB1 binding sites in the RhoC promoter.

Specific Aim4: To determine the distribution and stability of RhoC GTPase transcription variants in altering the half-life of the different mRNAs, thereby, regulating the total RhoC GTPase protein expression.

- Established RhoC and GAPDH probes for northern blot analysis.

Reportable outcomes:

- Established stable shRNA knockdown cell lines for FoxP3, HoxA3, HoxB7, HoxB8, HoxD9, HoxD10, CREB and NFκB1 in SUM149 cells.
- Developed a 4.0kbp RhoC promoter reporter
- Published a review titled, “Translocations in epithelial cancers.” (9)
- Published a manuscript detailing the methodology for identification of gene fusions in epithelial cancers, “Chimeric transcript discovery by paired-end transcriptome sequencing.” (8)
- A manuscript was accepted for publication at *Mol. Cancer Res*, “RhoC Expression and Head and Neck Cancer Metastasis” (*In Press*)
- Completed a book chapter that was accepted for publication, “The Rho GTPases in Cancer” (*In Press*)

Conclusions:

Since the submission of the original application and initiation of the DOD breast cancer training program, I have completed the core courses in Genetics, Biochemistry, Cell Biology and Ethics required by the University's CMB program as well as comprehensive courses in Cancer Biology, Pharmacology, Proteomics, Bioinformatics of Sequence Alignment and Mathematical Models in Biology. I have completed a comprehensive preliminary exam on a subject unrelated to this DOD award (my thesis project) as required by the CMB program. Additionally, I have been first author or co-author on three manuscripts and one book chapter accepted for publication on work directly disseminating from this DOD Breast cancer award.

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Chimeric transcript discovery by paired-end transcriptome sequencing

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Communicated by David Ginsburg, University of Michigan Medical School, Ann Arbor, MI, May 4, 2009 (received for review March 16, 2009)

Recurrent gene fusions are a prevalent class of mutations arising from the juxtaposition of 2 distinct regions, which can generate novel functional transcripts that could serve as valuable therapeutic targets in cancer. Therefore, we aim to establish a sensitive, high-throughput methodology to comprehensively catalog functional gene fusions in cancer by evaluating a paired-end transcriptome sequencing strategy. Not only did a paired-end approach provide a greater dynamic range in comparison with single read based approaches, but it clearly distinguished the high-level “driving” gene fusions, such as *BCR-ABL1* and *TMPRSS2-ERG*, from potential lower level “passenger” gene fusions. Also, the comprehensiveness of a paired-end approach enabled the discovery of 12 previously undescribed gene fusions in 4 commonly used cell lines that eluded previous approaches. Using the paired-end transcriptome sequencing approach, we observed read-through mRNA chimeras, tissue-type restricted chimeras, converging transcripts, diverging transcripts, and overlapping mRNA transcripts. Last, we successfully used paired-end transcriptome sequencing to detect previously undescribed ETS gene fusions in prostate tumors. Together, this study establishes a highly specific and sensitive approach for accurately and comprehensively cataloging chimeras within a sample using paired-end transcriptome sequencing.

bioinformatics | gene fusions | prostate cancer | breast cancer | RNA-Seq

One of the most common classes of genetic alterations is gene fusions, resulting from chromosomal rearrangements (1). Intriguingly, >80% of all known gene fusions are attributed to leukemias, lymphomas, and bone and soft tissue sarcomas that account for only 10% of all human cancers. In contrast, common epithelial cancers, which account for 80% of cancer-related deaths, can only be attributed to 10% of known recurrent gene fusions (2–4). However, the recent discovery of a recurrent gene fusion, *TMPRSS2-ERG*, in a majority of prostate cancers (5, 6), and *EML4-ALK* in non-small-cell lung cancer (NSCLC) (7), has expanded the realm of gene fusions as an oncogenic mechanism in common solid cancers. Also, the restricted expression of gene fusions to cancer cells makes them desirable therapeutic targets. One successful example is imatinib mesylate, or Gleevec, that targets *BCR-ABL1* in chronic myeloid leukemia (CML) (8–10). Therefore, the identification of novel gene fusions in a broad range of cancers is of enormous therapeutic significance.

The lack of known gene fusions in epithelial cancers has been attributed to their clonal heterogeneity and to the technical limitations of cytogenetic analysis, spectral karyotyping, FISH, and microarray-based comparative genomic hybridization (aCGH). Not surprisingly, *TMPRSS2-ERG* was discovered by circumventing these limitations through bioinformatics analysis of gene expression data to nominate genes with marked overexpression, or outliers, a signature of a fusion event (6). Building on this success, more recent strategies have adopted unbiased high-throughput approaches, with increased resolution, for genome-wide detection of chromosomal rearrangements in cancer involving BAC end sequencing (11), fosmid paired-end sequences (12), serial analysis of gene expression

(SAGE)-like sequencing (13), and next-generation DNA sequencing (14). Despite unveiling many novel genomic rearrangements, solid tumors accumulate multiple nonspecific aberrations throughout tumor progression; thus, making causal and driver aberrations indistinguishable from secondary and insignificant mutations, respectively.

The deep unbiased view of a cancer cell enabled by massively parallel transcriptome sequencing has greatly facilitated gene fusion discovery. As shown in our previous work, integrating long and short read transcriptome sequencing technologies was an effective approach for enriching “expressed” fusion transcripts (15). However, despite the success of this methodology, it required substantial overhead to leverage 2 sequencing platforms. Therefore, in this study, we adopted a single platform paired-end strategy to comprehensively elucidate novel chimeric events in cancer transcriptomes. Not only was using this single platform more economical, but it allowed us to more comprehensively map chimeric mRNA, hone in on driver gene fusion products due to its quantitative nature, and observe rare classes of transcripts that were overlapping, diverging, or converging.

Results

Chimera Discovery via Paired-End Transcriptome Sequencing. Here, we employ transcriptome sequencing to restrict chimera nominations to “expressed sequences,” thus, enriching for potentially functional mutations. To evaluate massively parallel paired-end transcriptome sequencing to identify novel gene fusions, we generated cDNA libraries from the prostate cancer cell line VCaP, CML cell line K562, universal human reference total RNA (UHR; Stratagene), and human brain reference (HBR) total RNA (Ambion). Using the Illumina Genome Analyzer II, we generated 16.9 million VCaP, 20.7 million K562, 25.5 million UHR, and 23.6 million HBR transcriptome mate pairs (2×50 nt). The mate pairs were mapped against the transcriptome and categorized as (i) mapping to same gene, (ii) mapping to different genes (chimera candidates), (iii) nonmapping, (iv) mitochondrial, (v) quality control, or (vi) ribosomal (Table S1). Overall, the chimera candidates represent a minor fraction of the mate pairs, comprising $\approx <1\%$ of the reads for each sample.

We believe that a paired-end strategy offers multiple advantages over single read based approaches such as alleviating the reliance on sequencing the reads traversing the fusion junction, increased coverage provided by sequencing reads from the ends of a tran-

Author contributions: C.A.M. and A.M.C. designed research; C.A.M., N.P., J.C.B., X.C., S.L., I.K., T.R.B., R.J.L., G.S., C.K.-S., and A.M.C. performed research; C.A.M., S.L., I.K., R.J.L., and G.S. contributed new reagents/analytic tools; C.A.M., N.P., J.C.B., S.K.-S., C.G., J.Y., R.J.L., G.S., C.K.-S., and A.M.C. analyzed data; and C.A.M., N.P., X.C., C.K.-S., and A.M.C. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0904720106/DCSupplemental.

scribed fragment, and the ability to resolve ambiguous mappings (Fig. S1). Therefore, to nominate chimeras, we leveraged each of these aspects in our bioinformatics analysis. We focused on both mate pairs encompassing and/or spanning the fusion junction by analyzing 2 main categories of sequence reads: chimera candidates and nonmapping (Fig. S24). The resulting chimera candidates from the nonmapping category that span the fusion boundary were merged with the chimeras found to encompass the fusion boundary revealing 119, 144, 205, and 294 chimeras in VCaP, K562, HBR, and UHR, respectively.

Comparison of a Paired-End Strategy Against Existing Single Read Approaches. To assess the merit of adopting a paired-end transcriptome approach, we compared the results against existing single read approaches. Although current RNA sequencing (RNA-Seq) studies have been using 36-nt single reads (16, 17), we increased the likelihood of spanning a fusion junction by generating 100-nt long single reads using the Illumina Genome Analyzer II. Also, we chose this length because it would facilitate a more comparable amount of sequencing time as required for sequencing both 50-nt mate pairs. In total, we generated 7.0, 59.4, and 53.0 million 100-nt transcriptome reads for VCaP, UHR, and HBR, respectively, for comparison against paired-end transcriptome reads from matched samples.

Because the UHR is a mixture of cancer cell lines, we expected to find numerous previously identified gene fusions. Therefore, we first assessed the depth of coverage of a paired-end approach against long single reads by directly comparing the normalized frequency of sequence reads supporting 4 previously identified gene fusions [*TMPRSS2-ERG* (5, 6), *BCR-ABL1* (18), *BCAS4-BCAS3* (19), and *ARFGF2-SULF2* (20)]. As shown in Fig. 14, we observed a marked enrichment of paired-end reads compared with long single reads for each of these well characterized gene fusions.

We observed that *TMPRSS2-ERG* had a >10-fold enrichment between paired-end and single read approaches. The schematic representation in Fig. 1B indicates the distribution of reads confirming the *TMPRSS2-ERG* gene fusion from both paired-end and single read sequencing. As expected, the longer reads improve the number of reads spanning known gene fusions. For example, had we sequenced a single 36-mer (shown in red text), 11 of the 17 chimeras, shown in the bottom portion of the long single reads, would not have spanned the gene fusion boundary, but instead, would have terminated before the junction and, therefore, only aligned to *TMPRSS2*. However, despite the improved results only 17 chimeric reads were generated from 7.0 million long single read sequences. In contrast, paired-end sequencing resulted in 552 reads supporting the *TMPRSS2-ERG* gene fusion from ≈17 million sequences.

Because we are using sequence based evidence to nominate a chimera, we hypothesized that the approach providing the maximum nucleotide coverage is more likely to capture a fusion junction. We calculated an *in silico* insert size for each sample using mate pairs aligning to the same gene, and found the mean insert size of ≈200 nt. Then, we compared the total coverage from single reads (coverage is equivalent to the total number of pass filter reads against the read length) with the paired-end approach (coverage is equivalent to the sum of the insert size with the length of each read) (Fig. S2B). Overall, we observed an average coverage of 848.7 and 757.3 MB using single read technology, compared with 2,553.3 and 2,363 MB from paired-end in UHR and HBR, respectively. This increase in ≈3-fold coverage in the paired-end samples compared with the long read approach, per lane, could explain the increased dynamic range we observed using a paired-end strategy.

Next we wanted to identify chimeras common to both strategies. The long read approach nominated 1,375 and 1,228 chimeras, whereas with a paired-end strategy, we only nominated 225 and 144 chimeras in UHR and HBR, respectively. As shown in the Venn diagram (Fig. 1C), there were 32 and 31 candidates common to both

technologies for UHR and HBR, respectively. Within the common UHR chimeric candidates, we observed previously identified gene fusions *BCAS4-BCAS3*, *BCR-ABL1*, *ARFGF2-SULF2*, and *RPS6KB1-TMEM49* (13). The remaining chimeras, nominated by both approaches, represent a high fidelity set. Therefore, to further assess whether a paired-end strategy has an increased dynamic range, we compared the ratio of normalized mate pair reads against single reads for the remaining chimeras common to both technologies. We observed that 93.5 and 93.9% of UHR and HBR candidates, respectively, had a higher ratio of normalized mate pair reads to single reads (Table S2), confirming the increased dynamic range offered by a paired-end strategy. We hypothesize that the greater number of nominated candidates specific to the long read approach represents an enrichment of false positives, as observed when using the 454 long read technology (15, 21).

Paired-End Approach Reveals Novel Gene Fusions. We were interested in determining whether the paired-end libraries could detect novel gene fusions. Among the top chimeras nominated from VCaP, HBR, UHR, and K562, many were already known, including *TMPRSS2-ERG*, *BCAS4-BCAS3*, *BCR-ABL1*, *USP10-ZDHHHC7*, and *ARFGF2-SULF2*. Also ranking among these well known gene fusions in UHR was a fusion on chromosome 13 between *GAS6* and *RASA3* (Fig. S34 and Table S2). The fact that *GAS6-RASA3* ranked higher than *BCR-ABL1* suggests that it may be a driving fusion in one of the cancer cell lines in the RNA pool.

Another observation was that there were 2 candidates among the top 10 found in both UHR and K562. This observation was intriguing, because hematological malignancies are not considered to have multiple gene fusion events. In addition to *BCR-ABL1*, we were able to detect a previously undescribed interchromosomal gene fusion between exon 23 of *NUP214* located at chromosome 9q34.13 with exon 2 of *XKR3* located at chromosome 22q11.1. Both of these genes reside on chromosome 22 and 9 in close proximity to *BCR* and *ABL1*, respectively (Fig. S3B). We confirmed the presence of *NUP214-XKR3* in K562 cells using qRT-PCR, but were unable to detect it across an additional 5 CML cell lines tested (SUP-B15, MEG-01, KU812, GDM-1, and Kasumi-4) (Fig. S3C). These results suggest that *NUP214-XKR3* is a “private” fusion that originated from additional complex rearrangements after the translocation that generated *BCR-ABL1* and a focal amplification of both gene regions.

Although we were able to detect *BCR-ABL1* and *NUP214-XKR3* in both UHR and K562, there was a marked reduction in the mate pairs supporting these fusions in UHR. Although a diluted signal is expected, because UHR is pooled samples, it provides evidence that pooling samples can serve as a useful approach for nominating top expressing chimeras, and potentially enrich for “driver” chimeras.

Previously Undescribed Prostate Gene Fusions. Our previous work using integrative transcriptome sequencing to detect gene fusions in cancer revealed multiple gene fusions, demonstrating the complexity of the prostate transcriptomes of VCaP and LNCaP (15). Here, we exploit the comprehensiveness of a paired-end strategy on the same cell lines to reveal novel chimeras. In the circular plot shown in Fig. S44, we displayed all experimentally validated paired-end chimeras in the larger red circle. We found that all of the previously discovered chimeras in VCaP and LNCaP comprised a subset of the paired-end candidates, as displayed in the inner black circle.

As expected, *TMPRSS2-ERG* was the top VCaP candidate. In addition to “rediscovering” the *USP10-ZDHHHC7*, *HJURP-INPP4A*, and *EIF4E2-HJURP* gene fusions, a paired-end approach revealed several previously undescribed gene fusions in VCaP. One such example was an interchromosomal gene fusion between *ZDHHHC7*, on chromosome 16, with *ABCB9*, residing on chromosome 12, that was validated by qRT-PCR (Fig. S3D). Interestingly, the 5' partner, *ZDHHHC7*, had previously been validated as a complex intrachro-

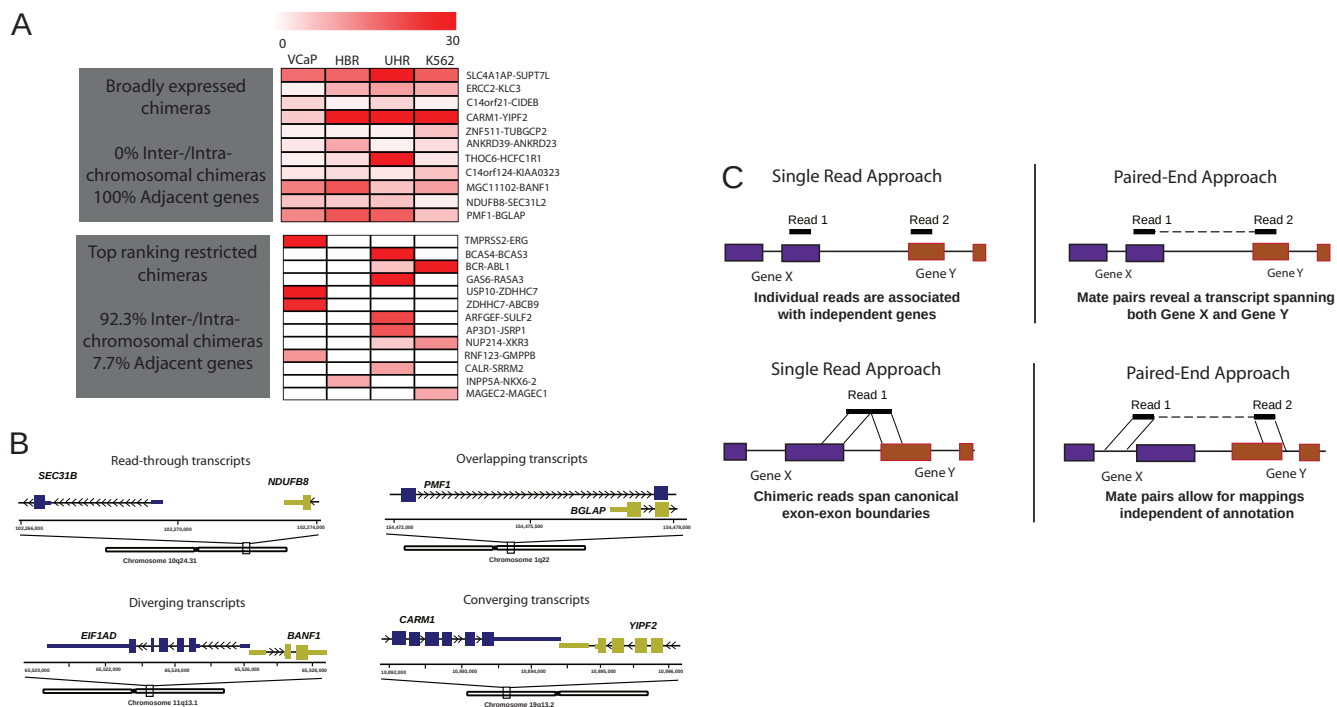


Fig. 2. RNA based chimeras. (A) Heatmaps showing the normalized number of reads supporting each read-through chimera across samples ranging from 0 (white) to 30 (red). (Upper) The heatmap highlights broadly expressed chimeras in UHR, HBR, VCaP, and K562. (Lower) The heatmap highlights the expression of the top ranking restricted gene fusions that are enriched with interchromosomal and intrachromosomal rearrangements. (B) Illustrative examples classifying RNA-based chimeras into (i) read-throughs, (ii) converging transcripts, (iii) diverging transcripts, and (iv) overlapping transcripts. (C Upper) Paired-end approach links reads from independent genes as belonging to the same transcriptional unit (Right), whereas a single read approach would assign these reads to independent genes (Left). (Lower) The single read approach requires that a chimera span the fusion junction (Left), whereas a paired-end approach can link mate pairs independent of gene annotation (Right).

level “driving” gene fusions, such as known recurrent gene fusions *BCR-ABL1* and *TPRSS2-ERG*, from lower level “passenger” fusions. Therefore, we plotted the normalized mate pair coverage at the fusion boundary for all experimentally validated gene fusions for the 2 cell lines that we sequenced harboring recurrent gene fusions, VCaP and K562. As shown in Fig. S4B, we observed that both driver fusions, *TPRSS2-ERG* and *BCR-ABL1*, show the highest expression among the validated chimeras in VCaP and K562, respectively. This observation suggests a paired-end nomination strategy for selecting putative driver gene fusions among private nonspecific gene fusions that lack detectable levels of expression across a panel of samples (15).

Previously Undescribed Breast Cancer Gene Fusions. Our ability to detect previously undescribed prostate gene fusions in VCaP and LNCaP demonstrated the comprehensiveness of paired-end transcriptome sequencing compared with an integrated approach, using short and long transcriptome reads. Therefore, we extended our paired-end analysis by using breast cancer cell line MCF-7, which has been mined for fusions using numerous approaches such as expressed sequence tags (ESTs) (22), array CGH (23), single nucleotide polymorphism arrays (24), gene expression arrays (25), end sequence profiling (20, 26), and paired-end diTag (PET) (13).

A histogram (Fig. S4C) of the top ranking MCF-7 candidates highlights *BCAS4-BCAS3* and *ARFGEF-SULF2* as the top 2 ranking candidates, whereas other previously reported candidates, such as *SULF2-PRICKLE*, *DEPDC1B-ELOVL7*, *RP56KB1-TMEM49*, and *CXorf15-SYAP1*, were interspersed among a comprehensive list of previously undescribed putative chimeras. To confirm that these previously undescribed nominations were not false positives, we experimentally validated 2 interchromosomal and 3 intrachromosomal candidates using qRT-PCR (Fig. S6). Overall, not only was

a paired-end approach able to detect gene fusions that have eluded numerous existing technologies, it has revealed 5 previously undescribed mutations in breast cancer.

RNA-Based Chimeras. Although many of the inter and intrachromosomal rearrangements that we nominated were found within a single sample, we observed many chimeric events shared across samples. We identified 11 chimeric events common to UHR, VCaP, K562, and HBR (Table S3). Via heatmap representation (Fig. 2A) of the normalized frequency of mate pairs supporting each chimeric event, we can observe these events are broadly transcribed in contrast to the top restricted chimeric events. Also, we found that 100% of the broadly expressed chimeras resided adjacent to one another on the genome, whereas only 7.7% of the restricted candidates were neighboring genes. This discrepancy can be explained by the enrichment of inter and intrachromosomal rearrangements in the restricted set.

Unlike, previously characterized restricted read-throughs, such as *SLC45A3-ELK4* (15), which are found adjacent to one another, but in the same orientation, we found that the majority of the broadly expressed chimera candidates resided adjacent to one another in different orientations. Therefore, we have categorized these events as (i) read-throughs, adjacent genes in the same orientation, (ii) diverging genes, adjacent genes in opposite orientation whose 5' ends are in close proximity, (iii) convergent genes, adjacent genes in opposite orientation whose 3' ends are in close proximity, and (iv) overlapping genes, adjacent genes who share common exons (Fig. 2B). Based on this classification, we found 1 read-through, 2 convergent genes, 6 divergent genes, and 2 overlapping genes. Also, we found that $\approx 81.8\%$ of these chimeras had at least 1 supporting EST, providing independent confirmation of the event (Table S3). In contrast to paired-end, single read ap-

One of the major advantages of using a transcriptome approach is that it enables us to identify rearrangements that are not detectable at the DNA level. For example, conventional cytogenetic methods would miss gene fusions produced by paracentric inversions, or sub microscopic events, such as *GAS6-RAS43*. Also, transcriptome sequencing can unveil RNA chimeras, lacking DNA aberrations, as demonstrated by the discovery of a recurrent, prostate specific, read-through of *SLC45A3* with *ELK4* in prostate cancers. Further classification of RNA based events using paired-end sequencing revealed numerous broadly expressed chimeras between adjacent genes. Although these events were not necessarily read-throughs events, because they typically had different orientations, we believe they represent extensions of transcriptional units beyond their annotated boundaries. Unlike single read based approaches, which require chimeras to span exon boundaries of independent genes, we were able to detect these events using paired-end sequencing, which could have significant impact for improving how we annotate transcriptional units.

Overall, we have demonstrated the advantages of employing a paired-end transcriptome strategy for chimera discovery, established a methodology for mining chimeras, and extensively catalogued chimeras in a prostate and hematological cancer models. We believe that the sensitivity of this approach will be of broad impact and significance for revealing novel causative gene fusions in various cancers while revealing additional private gene fusions that may contribute to tumorigenesis or cooperate with driver gene fusions.

Methods

Paired-End Gene Fusion Discovery Pipeline. Mate pair transcriptome reads were mapped to the human genome (hg18) and Refseq transcripts, allowing up to 2 mismatches, using Efficient Alignment of Nucleotide Databases (ELAND) pair within the Illumina Genome Analyzer Pipeline software. Illumina export output files were parsed to categorize passing filter mate pairs as (i) mapping to the same transcript, (ii) ribosomal, (iii) mitochondrial, (iv) quality control, (v) chimera candidates, and (vi) nonmapping. Chimera candidates and nonmapping categories were used for gene fusion discovery. For the chimera candidates category, the following criteria were used: (i) mate pairs must be of high mapping quality (best unique match across genome), (ii) best unique mate pairs do not have a more logical alternative combination (i.e., best mate pairs suggest an interchromosomal rearrangement, whereas the second best mapping for a mate reveals the pair have an alignment within the expected insert size), (iii) the sum of the distances between the most 5' and 3' mate on both partners of the gene fusion must be <500 nt, and (iv) mate pairs supporting a chimera must be nonredundant.

In addition to mining mate pairs encompassing a fusion boundary, the non-mapping category was mined for mate pairs that had 1 read mapping to a gene, whereas its corresponding read fails to align, because it spans the fusion boundary. First, the annotated transcript that the "mapping" mate pair aligned against was extracted, because this transcript represents one of the potential partners involved in the gene fusion. The "nonmapping" mate pair was then aligned against all of the exon boundaries of the known gene partner to identify a perfect partial alignment. A partial alignment confirms that the nonmapping mate pair maps to our expected gene partner while revealing the portion of the nonmapping mate pair, or overhang, aligning to the unknown partner. The overhang is then aligned against the exon boundaries of all known transcripts to identify the fusion partner. This process is done using a Perl script that extracts all possible University of California Santa Cruz (UCSC) and Refseq exon boundaries looking for a single perfect best hit.

Mate pairs spanning the fusion boundary are merged with mate pairs encompassing the fusion boundary. At least 2 independent mate pairs are required to support a chimera nomination, which can be achieved by (i) 2 or more nonredundant mate pairs spanning the fusion boundary, (ii) 2 or more nonredundant mate pairs encompassing a fusion boundary, or (iii) 1 or more mate pairs encompassing a fusion boundary and 1 or more mate pairs spanning the fusion boundary. All chimera nominations were normalized based on the cumulative number of mate pairs encompassing or spanning the fusion junction per million mate pairs passing filter.

RNA Chimera Analysis. Chimeras found from UHR, HBR, VCaP, and K562 were grouped based on whether they showed expression in all samples, "broadly expressed," or a single sample, "restricted expression." Because UHR is comprised of K562, chimeras found in only these 2 samples were also considered as restricted. Heatmap visualization was conducted by using TIGR's MultiExperiment Viewer (TMeV) version 4.0 (www.tm4.org).

Additional Details. Additional details can be found in [SI Text](#).

ACKNOWLEDGMENTS. We thank Lu Zhang, Eric Vermaas, Victor Quijano, and Juying Yan for assistance with sequencing, Shawn Baker and Steffen Durinck for helpful discussions, Rohit Mehra and Javed Siddiqui for collecting tissue samples, and Bo Han and Kalpana Ramnarayanan for technical assistance. C.A.M. was supported by a National Institutes of Health (NIH) Ruth L. Kirschstein postdoctoral training grant, and currently derives support from the American Association of Cancer Research Amgen Fellowship in Clinical/Translational Research and the Canary Foundation and American Cancer Society Early Detection Postdoctoral Fellowship. J.Y. was supported by NIH Grant 1K99CA129565-01A1 and Department of Defense (DOD) Grant PC080665. A.M.C. was supported in part by the NIH (Prostate SPORE P50CA69568, R01 R01CA132874), the DOD (BC075023, W81XWH-08-0110), the Early Detection Research Network (U01 CA111275), a BurroughsWellcome Foundation Award in Clinical Translational Research, a Doris Duke Charitable Foundation Distinguished Clinical Investigator Award, and the Howard Hughes Medical Institute. This work was also supported by National Center for Integrative Biomedical Informatics Grant U54 DA021519.

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Review

Translocations in epithelial cancers

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ARTICLE INFO

Article history:

Received 25 February 2009

Accepted 21 April 2009

Available online 3 May 2009

Keywords:

Translocation

Epithelial

Rearrangement

Gene fusion

Chimera

MLL

ERG

ALK

HMGA2

COPA

ABSTRACT

Genomic translocations leading to the expression of chimeric transcripts characterize several hematologic, mesenchymal and epithelial malignancies. While several gene fusions have been linked to essential molecular events in hematologic malignancies, the identification and characterization of recurrent chimeric transcripts in epithelial cancers has been limited. However, the recent discovery of the recurrent gene fusions in prostate cancer has sparked a revitalization of the quest to identify novel rearrangements in epithelial malignancies. Here, the molecular mechanisms of gene fusions that drive several epithelial cancers and the recent technological advances that increase the speed and reliability of recurrent gene fusion discovery are explored.

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1. Introduction

Throughout history, technological advances are often followed by discoveries that dramatically alter our perceptions of disease etiology. For example, after the term “chromosome” was introduced in the mid-1849's, several German pathologists began using techniques to compare gross mitotic changes in tissue sections from different human malignancies [1]. Almost half of a century later, Theodore Boveri published a critical hypothesis that, “mammalian tumors might be initiated by mitotic abnormalities that resulted in a change in the number of chromosomes in the cell (aneuploidy)”, based on the observation that sea urchin embryos would frequently engage in uncommon development following mitotic abnormality [2]. As time passed, breakthroughs arose that dramatically increased the quality and reproducibility of cytogenetic techniques such as the use of colchicine, which arrests cells in mitosis by inhibiting microtubule assembly. As a result of these observations, the general hypotheses regarding the evolution of human disease became increasingly complex; particular pathological conditions were associated with specific chromosomal abnormalities, such as Lejeune's association of Down syndrome with an extra copy of chromosome 21 [3,4].

Advances in technology once again spurred discovery when, in 1958, Rothfels and Siminovitch published a new cytogenetic, air-drying technique for flattening chromosomes [5]. The application of this technology later allowed Hungerford and Nowell to further characterize their initial observation that two patients with chronic myelogenous leukemia (CML) had a characteristic small chromosome [6]. Soon after the initial publication, Hungerford and Nowell were able to report on a series of seven patients, all of which harbored this minute chromosome [7]. This was coined the “Philadelphia chromosome” after the city in which the abnormal chromosome was discovered in accord with the Committee for the Standardization of Chromosomes [8]. The rearrangement leading to the Philadelphia chromosome was eventually characterized as a translocation between chromosomes 9 and 22 [9], resulting in the fusion of the breakpoint cluster region (BCR) gene on chromosome 22 with the v-abl Abelson murine leukemia viral oncogene homolog (ABL1) gene on chromosome 9 [10]. Later in 1990, Lugo et al. demonstrated that the BCR–ABL1 fusion protein is an active tyrosine kinase, through immunoblotting cell lysates from Rat 1 transfected cells, revealing that cells transfected with either BCR–ABL1 or v-src, but not v-H-ras or v-myc, had a significant increase in total phosphotyrosine content [11]. Understanding the molecular mechanism of BCR–ABL1 led to the development of one of the first molecularly tailored therapies as the small molecule Imatinib was specifically selected for its ability to inhibit BCR–ABL1 kinase activity [12,13]. The success of treating chronic myelogenous leukemia with a specific inhibitor of the BCR–ABL1 chimera led to a strong interest in the discovery of novel gene fusions in other cancer subtypes with the long term goal of designing disease specific therapeutics.

As techniques like the use of chromosome banding for karyotypic analysis were improved, the impact on discovery of novel gene fusions was immediately evident in leukemias and lymphomas. In fact, while BCR–ABL1 is perhaps the most famous gene fusion, the first molecularly characterized chimera was discovered by Zech et al. through the use of karyotypic analysis and is actually involved in the pathogenesis of Burkett's lymphoma and was identified. While this karyotypic analysis demonstrated absence of the distal region on the long arm of chromosome 8 and an extra band in the long arm chromosome 14 distal segment [14], the genes involved in the

rearrangement remained elusive until 1982 when it was demonstrated that the translocation altered the c-MYC oncogene [15] and that the promoter and 5' region of the immunoglobulin heavy chain (IGH) gene were rearranged such that the IGH promoter controls c-MYC expression [16]. Although this fusion does not lead to a chimeric protein, it was demonstrated that aberrant c-MYC expression through the IGH promoter is a necessary component of malignant transformation in Burkett's lymphoma [17].

As with lymphoma research, karyotypic analysis rapidly led to the identification of recurrent breakpoints that seemed to characterize subsets of myeloid leukemia. For example, in 1973, the acute myeloid leukemia 1 (AML1) gene was cloned from the breakpoint region of the first recurrent translocation described in leukemia, t(8;21) [18]. In 1991, the AML1 gene was found to be fused to the eight-twenty one (ETO) gene on chromosome 21, which is also known as runt-related transcription factor 1 translocated to 1 (RUNX1T1) [19,20].

As the techniques of molecular biology improved, it became easier and easier to obtain the DNA sequence adjacent to chromosomal breakpoints. Since the original identification of AML1 in myeloid leukemia, over 10 genes have been described to participate in rearrangements with AML1 [21]. In fact, advances in sequencing technology led to the realization that several genes are recurrently and promiscuously fused to multiple partners; the examples of which are ever increasing. In addition to AML1, the other notable example of a promiscuous fusion gene partner is the mixed-lineage leukemia (MLL) gene, which is involved in over 40 different rearrangements (reviewed in [22]). In fact, because of the variety and difficulty of discussing all chromosomal aberrations in human malignancies, Mitelman et al. maintain and frequently update an online database of rearrangements and chromosome aberrations from all malignant neoplasms [23].

With the rapid development of current technologies like high-throughput sequencing, our perceptions as to the origins of disease have revealed a critical involvement of chromosomal aberrations, in particular, the role of translocations and gene fusions in malignant development. With a better understanding of the role of these chromosomal aberrations, therapies designed to inhibit the molecular function of chimeric proteins have recently been developed and, like Imatinib, some have demonstrated a window of strong efficacy. Consequently, much hope has been generated by the potential for targeting existing and novel gene fusions that characterize specific cancer subtypes with rationally designed molecularly tailored therapies. Here, we review known genomic rearrangements in epithelial tumors that led to aberrant expression of chimeric transcripts and the emerging technologies that may lead to the identification of novel gene fusions.

2. Gene fusions in epithelial cancers

In order to highlight the number of genomic rearrangements leading to fusion genes that characterize epithelial cancers, we have surveyed some of the well-studied chimeras from several solid malignancies and describe the fusions in approximate chronological order (Fig. 1). In the ensuing sections, we will analyze concepts from a global view of epithelial gene fusions with a few case studies of rearrangements from leukemia and endometrial stromal tumors. Gene fusions will be categorized into three different types: (1) those which alter the transcriptional regulation, (2) those which alter mRNA regulation and (3) those which alter protein activity. This will be followed by a discussion of the potential reasons why gene fusions have not been in the limelight of solid tumor pathogenesis and the

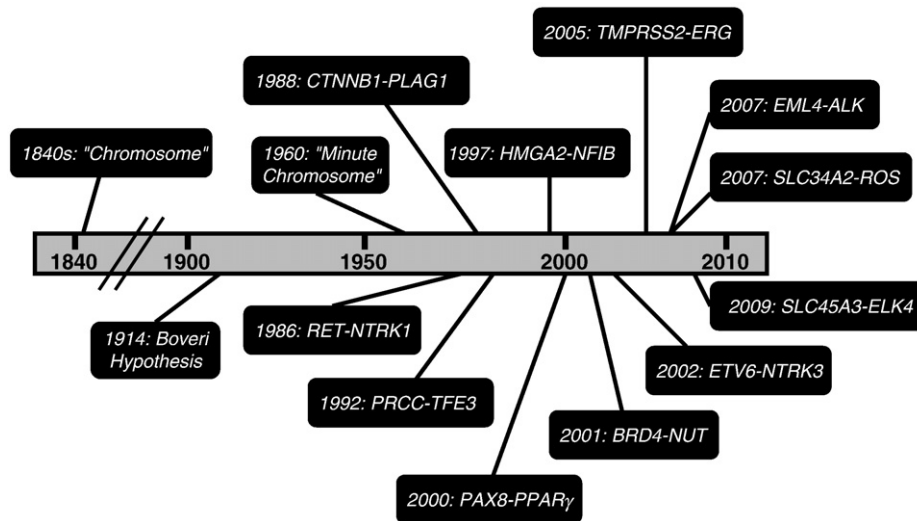


Fig. 1. Chronology of gene fusion discoveries in epithelial cancers.

developing technologies that are being used to find novel recurrent gene fusions in common epithelial tumors.

2.1. *RET-NTRK1*

The initial discovery of an epithelial gene fusion in mid-1989 comes directly from a novel screening technique used to identify transforming oncogenes. In this experimental approach, immortalized NIH3T3 cells were transfected with fragments of tumor cell genomic DNA, plated in soft agar. DNA is then isolated from cells and sequenced or sub-cloned to identify critical fragments. Using this approach, Martin-Zanca et al. identified the *RET-NTRK1* genomic translocation, providing some of the first insights into the possibility that recurrent genomic rearrangements were not specifically of hematologic phenomena [24].

RET (rearranged during transfection) encodes a tyrosine kinase [25,26] that was originally identified through transfection of DNA from a human T-cell lymphoma into NIH3T3 cells [27]. *NTRK1* is a membrane-bound tyrosine kinase receptor that regulates neuronal cell growth, differentiation, and programmed cell death pathways [28]. Fusion of these two genes results in loss of the *NTRK1* signal sequence giving rise to cytoplasmic localization and constitutive activation of the fusion protein [29]. Interestingly, although *NTRK1* was the first identified *RET* fusion partner, *RET* has several other N-terminal fusion partners including *H4* [30,31], *R1α* [32], *RFG5* [33] and *ELE1* [34,35]. One possible explanation for the diversity of genomic rearrangements observed in PTC is that the underlying pathology is simply dependent on deregulation of either the *RET* or *NTRK1* tyrosine kinase domain (reviewed in [36]). Consequently, the important determining event in PTC carcinogenesis may be constitutive activation of the mitogen-activated protein kinase (MAPK) signaling pathway, which can be caused by rearrangement of either the *RET* and/or *NTRK1* gene. One reason for this hypothesis is that while the *RET-NTRK1* rearrangement appears to be the predominant gene fusion responsible for childhood PTC, in adult-onset populations activating point mutations in the *BRAF* gene or, controversially, the *RAS* gene [37–43], also lead to constitutive activation of the MAPK pathway without *RET* and/or *NTRK1* genomic rearrangement [44].

In addition to differences in the age-related molecular onset of PTC, the proportion of cases with either a *RET* or *NTRK1* rearrangement also appears to be based on the geographic area of origin [45–47], possibly because thyroid cancer is established to be associated with exposure to ionizing radiation [37,48]. Indeed, studies of patient populations exposed to either the Chernobyl nuclear power plant accident [49,50]

or the atomic bombings [51] have demonstrated that genomic rearrangements occur at a higher frequency than mutations following extreme exposure to radiation [37,48], suggesting that under certain biological conditions exposure to high dose radiation may actually trigger specific DNA breaks leading to intentional genomic rearrangement. In fact, the fusion proteins that characterize PTC contain a number of different N-terminal partners fused the C-terminal tyrosine kinase domain of either *RET* or *NTRK1* [52] that may depend on the environmental cues leading to genomic rearrangement.

2.2. *CTNNB1-PLAG1*

Within a year of publication of the *RET-NTRK1* genomic rearrangement in PTC, another epithelial translocation was reported in pleomorphic adenoma (PA) [53], a slow-growing epithelial tumor that is responsible for more than 50% of salivary gland tumors [54], but less than 10% of tumors from the head and neck [55]. In contrast to *RET-NTRK1* which was discovered by a screening technique, rearrangements in PA were first identified by karyotypic analysis of primary tumors. In fact, before any of the breakpoint genes were identified, PAs were already divided into four cytogenetic groups (reviewed in [56]). Rearrangements of 8q12 account for about 40% of PAs with t(3;8)(p21;q12) comprising about half of rearrangements at this locus. Translocations of 12q14–15 account for about 8% of PAs with t(9;12)(p12–22;q13–15) or an ins(9;12)(p12–22;q13–15) responsible for these abnormalities [57,58]. Tumors with non-recurrent clonal changes comprise about 20% of PAs, and tumors with apparently normal karyotypes account for the remaining cases [56].

Almost 20 years after the initial karyotyping studies, Kas et al. used a comprehensive breakpoint mapping approach, southern blot analysis and 5' rapid amplification of cDNA ends (5' RACE) to identify the genes involved in the most prevalent PA translocation, t(3;8)(p21;q12) as *β-Catenin* (*CTNNB1*) and *PLAG1* (pleomorphic adenoma gene 1) [59]. Specifically, the t(3;8)(p21;q12) rearrangement fuses the *β-Catenin* (*CTNNB1*) promoter and exon 1 to *PLAG1* exon 2, resulting in a marked increase in *PLAG1* expression (Fig. 2). As such, because the gene fusion results in altered DNA level regulation of *PLAG1* transcript, this gene fusion is characterized as type 1. Interestingly, the reciprocal translocation links the *PLAG1* promoter and exon 1 to *β-Catenin* exon 2, reducing *β-Catenin* expression. As *β-Catenin* signals through several well-characterized oncogenic pathways (reviewed in [60]), the reduction in *β-Catenin* is curious. *PLAG1*, however, belongs to the *PLAG* family of proteins and encodes a zinc finger protein with two putative nuclear localization signals and can bind to either DNA or

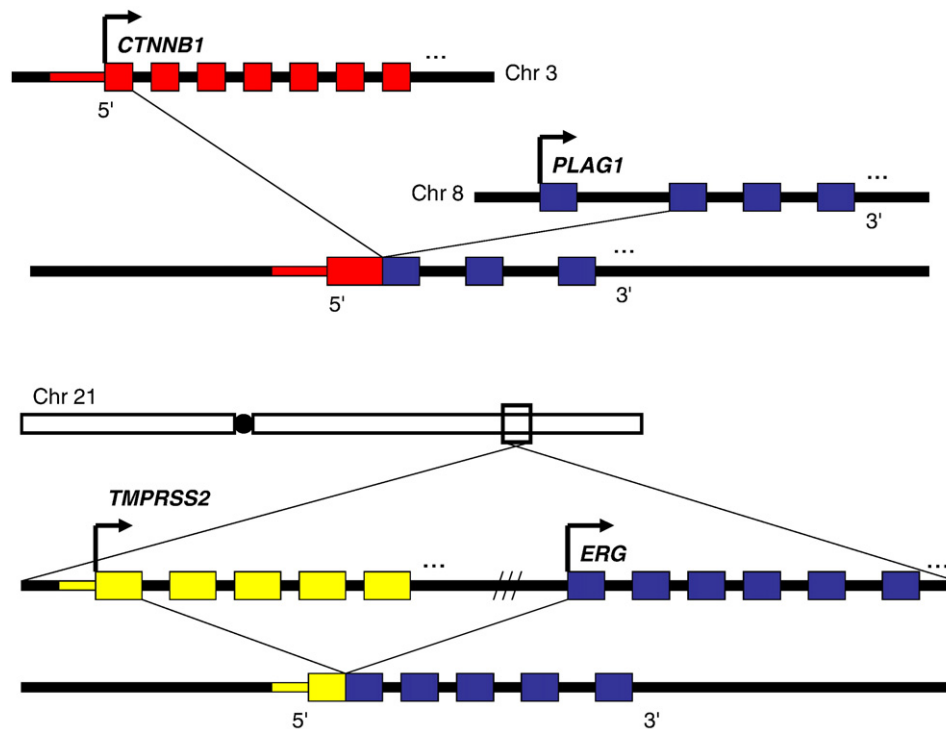


Fig. 2. Genomic structure of gene fusions with altered transcriptional regulation. The *CTNNB1-PLAG1* and *TMPRSS2-ERG* chimeras represent an important class of gene fusions in which the proto-oncogene remains largely intact, but the genomic rearrangement places a new promoter and 5'-UTR upstream of the main coding sequence, leading to aberrant expression of the proto-oncogene.

RNA. Forced expression of *PLAG1* in NIH3T3 cells has demonstrated that this protein can induce the standard characteristics of neoplastic transformation including loss cell–cell contact inhibition, anchorage-independent growth, and tumor formation in nude mice xenografts [61]. This suggests that the constitutive activity of the *CTNNB1* promoter leads to sufficient *PLAG1* expression for malignant transformation in PA.

2.3. *PRCC-TFE3*

As cloning and molecular strategies improved in the early 1990's, another recurrent gene fusion would soon be described in papillary renal cell carcinoma (PRCC), the second most common carcinoma of the renal tubules accounting for 15–20% of all renal cell carcinomas [62–66]. Karyotypic analysis as early as 1986 (de Jong et al.) led to the identification of abnormalities in the Xp11.22 region characterized by a genomic rearrangement, t(X;1)(p11.2;q21.2) [62–66]. Interestingly, before any of the genes surrounding the breakpoint were cloned a gene encoding *TFE3*, which was originally identified by their ability to bind to μ E3 elements in the immunoglobulin heavy chain intronic enhancer [67], was mapped to the Xp11.22 locus [68], and later shown to encode a member of the basic helix–loop–helix followed by a leucine zipper family (bHLHzip) of transcription factors. After the original genomic mapping, *TFE3* was soon identified at the translocation breakpoint by southern blot analysis [69]. Subsequent 5'-RACE identified *PRCC*; a ubiquitously expressed gene that encodes a protein with a high proportion of prolines and glycines – including three P-X-X-P motifs that are known to interact with SH3 domains [70,71]. Interestingly, the fusion event leading to the *PRCC-TFE3* rearrangement also results in a reciprocal *TFE3-PRCC* gene fusion [69,72].

To elucidate the properties of these reciprocal gene fusions, Weterman et al. introduced wild type *PRCC*, wild type *TFE3*, *PRCC-TFE3* and *TFE3-PRCC* expression vectors into COS cells and postulated that only the *PRCC-TFE3* gene fusion was responsible for tumor formation based on its ability to activate a generalized report assay

[73]. Thus, the *PRCC-TFE3* genomic rearrangement is type 3 as the fusion protein gained a novel function through rearrangement. However, fusions of the *PSF* or *NonO* pre-mRNA splicing factors are also recurrently fused to *TFE3*, albeit at a much lower frequency than *PRCC* [69,72,74], suggesting that the *TFE3* portion of the fusion is responsible for malignant transformation. Subsequent transcriptional activation assays demonstrated that of the *PSF-TFE3*, *NonO-TFE3* and *PRCC-TFE3* chimeras, only the *PRCC-TFE3* fusion protein could activate the plasminogen activator inhibitor-1 (*PAI-1*) promoter [75], suggesting that only this gene fusion retains transcriptional activity. However, recent co-immunoprecipitation experiments demonstrated that antibodies against the pre-mRNA splicing factors SC35, PRL1, and CDC5 were able to immunoprecipitate wild type *PRCC*, and an anti-SM antibody was able to immunoprecipitate the *PRCC-TFE3* fusion protein [75]. This data suggests that the fusion protein functions may partially function through transcriptional pathways, it may also function by altering pre-mRNA splicing, but more conclusive experiments need to be conducted to demonstrate this phenotype.

2.4. *HMG2*, evading *let-7*

While most of the gene fusions discovered until this point including *PRCC-TFE3* were thought to define specific epithelial tumor types, a new gene fusion that was associated with several different tumor types, including pleomorphic adenoma (PA) (see above), lipoma, uterine leiomyoma and some myeloid malignancies [76], would refute the notion. In fact, the discovery of translocations involving 12q15 had been established by karyotypic analysis in multiple tumor types before the rearranged genes were actually identified and one of the genes involved in the t(9;12)(p12–22;q13–15) PA translocation was first identified in both mesenchymal tumors [77] and lipomas [78]. This first gene to be described was the 5' gene fusion partner, *HMG2* (high mobility group AT-hook 2), belongs to the non-histone chromosomal high mobility group (HMG) protein family, which are small nuclear proteins (<30 kDa) that undergo

extensive post-translational modifications and contain nine amino acid segments that bind AT-rich DNA stretches in the minor groove (AT-hooks) (reviewed in [79]). Subsequent 3' RACE of tumor samples revealed that HMGA2 has two different 3' partners in PA, *FHIT* and *NFIB*, both of which contribute very little coding sequence to the resulting fusion gene. In fact, in one class of translocations, *HMGA2* exon 3 is fused to *FHIT* exon 9 or 10, resulting in retention of the C-terminal 26 amino acids of *FHIT* [80], and in the other set, *HMGA2* exon 3 or 4 fusion to *NFIB* exon 9 appends five amino acids (SWYLG) to the truncated *HMGA2* protein [81].

Surprisingly, transgenic mice overexpressing wild type *HMGA2* were observed to have similar phenotypes to mice expressing the truncated protein *HMGA2* protein found in the PA gene fusions [82–84]. To complicate this observation, in hereditary renal cell carcinoma, *FHIT* was previously demonstrated to be fused to the patched related gene *TRC8* by t(3;8)(p14.2;q24.1) [85,86] and the (SWYLG) amino acid motif found in the *HMGA2*–*NFIB* gene fusion were shown to be essential for *NFIB* function [81]. Recent research, however, has shed light onto the importance of these translocations to neoplastic transformation.

The discovery that small RNAs called microRNAs can negatively regulate gene expression through direct binding to a gene's 3'-UTR has led to the hypothesis that certain microRNAs can function as tumor suppressors in cancer [87]. Bioinformatic analysis of the *HMGA2* 3'-UTR demonstrated that the mRNA contains seven conserved sites complementary to the *let-7* microRNA [88] (depicted in Fig. 3). To show that the *let-7* microRNA negatively influences *HMGA2* expression, Mayr et al. built a *HMGA2* 3'-UTR conjugated

luciferase reporter and demonstrated that *let-7* represses its expression [89]. As such, although the genomic rearrangements between *HMGA2* and *FHIT* or *NFIB* yield fusion proteins, replacement of a *Let-7* regulated 3'-UTR seems to be the critical event because it leads to *HMGA2* overexpression, which is sufficient for neoplastic transformation. Thus, the *HMGA2* genomic rearrangement represent the first of a novel class of gene fusions, type 2, in which fusion gene activity is enhanced by loss of mRNA level regulation (Fig. 3).

2.5. Pax8–PPAR γ

In 2000, Kroll et al. employed fluorescence in situ hybridization (FISH), yeast artificial chromosome mapping and 3' RACE to identify genes involved in a genomic rearrangement, t(2;3)(q13;p25) [90], that was originally identified by karyotype analysis of follicular thyroid carcinomas, a subset (10–20%) of all thyroid malignancies [91]. This translocation is thought to be specific to FTC as it has not been reported in other thyroid tumors or hyperplastic nodules [92]. In the resulting gene fusion, the *Pax8* (Paired box gene 8) gene is fused to *PPAR γ* (Peroxisome proliferator-activated receptor- γ), a ubiquitously expressed transcription factor [90]. The *Pax8* protein is involved in thyroid follicular cell development and regulation of thyroid-specific gene expression [93]. *PPAR γ* plays a major role in a number of different diseases including obesity, atherosclerosis, diabetes as well as cancer (reviewed in [94]). Because *Pax8* is a thyroid-specific transcription factor and because its DNA binding domain is fused to the c-terminal domains of *PPAR γ* [90], the resulting protein chimera is thought to have constitutive re-distribution of *PPAR γ* -directed

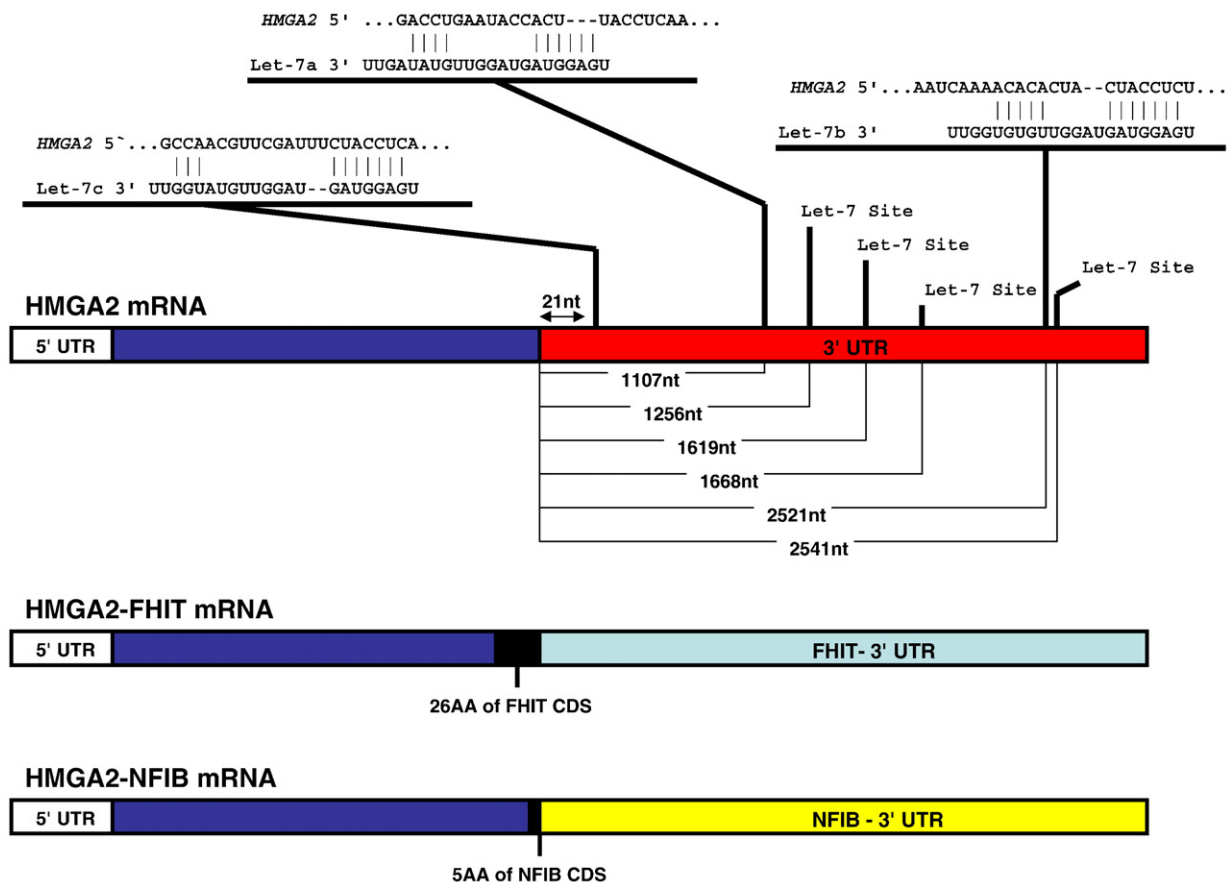


Fig. 3. *HMGA2* gene fusions elude the *Let-7* family of microRNAs. The *HMGA2* mRNA structure is shown along with putative *Let-7* family binding sequences in the *HMGA2* 3'-UTR. Results were predicted by TargetScan [202] and three representative microRNAs are shown with their highest probability binding sites of the seven total predicted sites along the 3' UTR. Distance to each predicted binding site is annotated as nucleotides from the start of the 3' UTR. Below the wild type *HMGA2* mRNA are the *HMGA2*–*FHIT* and *HMGA2*–*NFIB* mRNAs that result from these two gene fusions. TargetScan did not predict any microRNA binding sites in these genes. As such, the *HMGA2* gene fusions represent a second class of gene fusions in which the recombination event allows the proto-oncogene mRNA to evade microRNA-mediated silencing.

transcription. In 2005, gene-expression microarray profiling revealed a distinct signature in follicular thyroid carcinomas harboring the *Pax8-PPAR γ* gene fusion in which cell growth and chromatin remodeling pathways were over-represented and protein biosynthesis pathways were under-represented as compared to follicular thyroid carcinomas without the translocation [95], suggesting that *PPAR γ* -transcription is indeed redefined by the gene fusion.

Interestingly, follicular thyroid carcinomas were originally thought to arise from disruption of distinct molecular pathways, either through the fusion of *Pax8* to *PPAR γ* , or through the acquisition of point mutations leading to the constitutive activation of the G-protein RAS. In fact, one study reported that 16/33 (49%) of follicular carcinomas had *RAS* mutations, 12/33 (36%) had *Pax8-PPAR γ* rearrangement, only 1/33 (3%) had both, and 4/33 (12%) had neither [96]. However, in 2006, quantitative reverse transcription PCR analysis of follicular carcinoma clinical samples demonstrated loss of the tumor suppressor *NORE1A* in samples harboring the *Pax8-PPAR γ* rearrangement, but not in other samples [97]. Because *NORE1A* binds to the GTP bound (activated) RAS protein and suppresses RAS activity, this discovery suggested that activation of the RAS pathway is a critical event in pathogenesis of thyroid carcinoma that is altered either directly by activating mutation, or indirectly by the *Pax8-PPAR γ* rearrangement.

2.6. BRD–NUT

Soon after the discovery of the *Pax8-PPAR γ* rearrangement, the translocation t(15;19)(q13;p13.1) was identified in a rare, highly aggressive carcinoma arising in the midline organs and upper respiratory tract of young people now termed nuclear protein in testis (NUT) midline carcinomas (NMC) [98–100]. *BRD4*, which contains the chromosome 19 breakpoint, has two annotated transcripts encoding either short or long forms of the protein that both contain N-terminal bromodomains. The longer *BRD4* transcript encodes a ubiquitously expressed 200 kDa nuclear protein [101] with a c-terminal lysine rich region that is not found in the shorter transcript. The translocation resulting in fusion to the *NUT* gene (identified by southern blot analysis) only disrupts the longer *BRD4* transcript resulting in the loss of the lysine rich region in the fusion

oncogene. Several studies of *BRD4* in both murine and human cell line models have demonstrated a critical role in cell cycle progression and cell proliferation [102,103]. In fact, Brd4 enhances cell growth by interacting with chromatin [104], replication factor C [102] and cyclinT1 and CDK1 that constitute core positive transcription elongation factor b (P-TEFb) [105]. Likewise, chromatin immunoprecipitation assays demonstrated that Brd4 is required to recruit P-TEFb to active promoters, and that increased Brd4 leads to increased P-TEFb-dependent phosphorylation of RNA polymerase and enhanced transcription from promoters *in vivo* [105].

More insight into the role of the *BRD4-NUT* fusion protein in NMC biology came from a screen for other NMC gene fusions. Because the *BRD4-NUT* translocation defines two-thirds of all NMCs, French et al. used a candidate gene approach to screen other NMC samples and discovered another recurrent translocation between *BRD3* and *NUT* that defined large portion of the remaining NMC cases [106]. The *BRD3-NUT* fusion gene encodes a protein highly similar to that encoded by the *BRD4-NUT* transcript. It is composed of two tandem chromatin-binding bromodomains, an extra-terminal domain, a bipartite nuclear localization sequence, and a significant portion of *NUT* coding sequence. As such, the conserved protein structure gave insight into the mechanism by which the chimeric protein induces neoplastic properties.

Wild type *NUT*, which is normally only expressed in the testis [99], contains both nuclear localization and export signal sequences and is shuttled between the nucleus and cytoplasm via a leptomycin-sensitive pathway [106]. Importantly, however, the Brd3–*NUT* and Brd4–*NUT* proteins are retained in the nucleus, suggesting that interactions between the Brd3 or Brd4 bromodomains and chromatin are essential to the fusion protein [106] (Fig. 4). Further evidence for this hypothesis comes from an siRNA experiment in which knock-down of Brd–*NUT* fusion transcripts in NMC cell lines resulted in squamous differentiation and cell cycle arrest [106]. This suggested that the nuclear retention of *NUT*, not the loss of the Brd C-terminal domain, is responsible for promoting NMC carcinogenesis [106]. The realization that *Brd-NUT* gene fusions define a class of translocations that fuse bromodomains to the *NUT* protein suggests that oncogenic translocations will arise from multiple partners when critical domains are present in more than one gene.

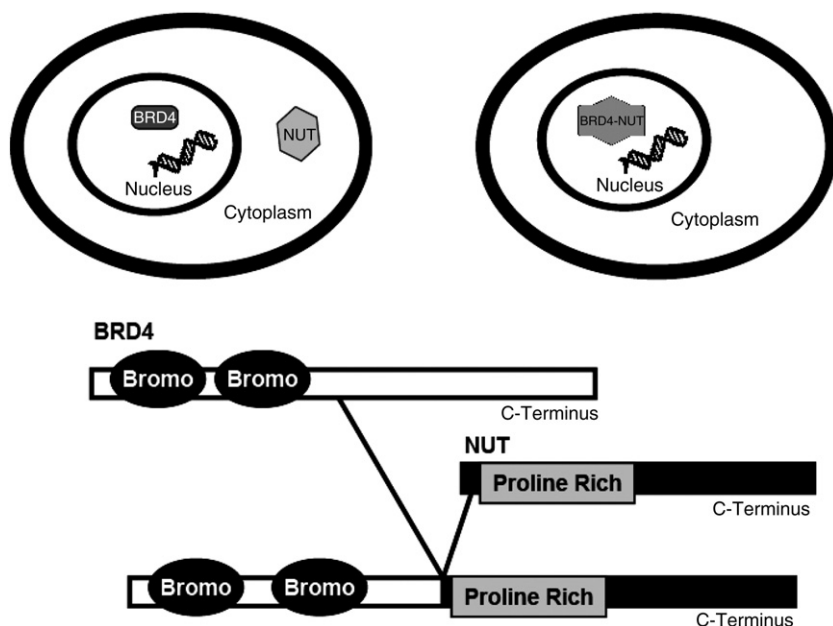


Fig. 4. Nuclear retention of NUT. The *BRD4-NUT* gene fusion represents a third class of rearrangements in which the resulting protein gains activity to become a proto-oncogene. In this case, the two bromodomains of *BRD4* are fused to *NUT*. Although *NUT* usually cycles between the nucleus and cytoplasm in a highly controlled manner, appendage of the *BRD4* bromodomains to the majority of the *NUT* protein lead to nuclear retention of the protein and aberrant activity.

2.7. ETV6–NTRK3

The first major example of a recurrent epithelial rearrangement that appeared not only in multiple tumor types, but had also been reported in a large subset of hematologic malignancies was detected in several cases of secretory breast carcinoma, a rare subtype of infiltrating ductal carcinoma affecting both children and adults [107]. Tognon et al. detected the *ETV6–NTRK3* fusion by comprehensive FISH analysis in 92% (12 of 13) secretory breast carcinoma cases [108]. *ETV6* (also *TEL*) is an ETS family member that is involved in a large number of fusions to either a transcription factor like *AML1* [109] or to a protein tyrosine kinase domain like that of *ABL* [110,111], *JAK2* [112–114], *ARG* [115,116], *PDGFR β* [117] or *FGFR3* [118], each of which define a unique leukemia subtype (reviewed in [119]). *ETV6* contains a pointed oligomerization domain (PNT; also known as sterile alpha motif, SAM, or helix–loop–helix, HLH) and an ETS DNA binding domain, the expression of which is required for developmental processes such as hematopoiesis and yolk sac angiogenesis [120]. *NTRK3* is a transmembrane neurotrophin-3 surface receptor that contains a c-terminal protein tyrosine kinase domain and plays a role in growth, development, and cell survival of neural cells in the central nervous system (reviewed in [121]). The fusion of the N-terminal *ETV6* pointed domain to the C-terminal tyrosine kinase domain of *NTRK3* was first reported in congenital fibrosarcoma (CFS) [122], but has since been reported in multiple cell lineages including those that give rise to congenital mesoblastic nephroma (CMN), acute myelogenous leukemia, and secretory breast carcinoma [108] (reviewed in [123]).

Following the initial discovery, research focused on the transforming ability of the recombination product. By using retroviral gene delivery methods, the *ETV6–NTRK3* fusion gene was shown to be sufficient to induce the non-tumorigenic murine breast cell lines Eph4 (epithelial) and Scg6 (myoepithelial) as well as NIH-3T3 fibroblasts to form tumors, glandular structures and to express epithelial antigens [108]. This discovery suggested that the fusion gene acts as a dominant oncogene in secretory breast cancer. *ETV6–NTRK3* was also shown to inhibit TGF- β tumor suppressor activity in NIH3T3 cells [124], suggesting that it most likely regulates microRNA biogenesis indirectly [125], but this has not yet been explored. Although it is known that adults have a less favorable prognosis than children and distant metastases are rare [126], local recurrences and nodal metastases have been observed [127] suggesting that the gene fusion leads to an invasion-associated transcriptional program, but this also has not been explored. Despite this, it is known that constitutive activation of the fusion protein leads to activation of the Ras-mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide-3-kinase (PI3K)–AKT pathway, the mechanism leading to activation of these pathways has remained elusive until recently, when the fusion protein was shown to associate with c-Src by immunoprecipitation from fusion-positive CFS and CMN human primary tumors [128]. More recently, however, a mouse knockin model was created by introducing the human *NTRK3* cDNA into exon 6 of the mouse *ETV6* locus, which induced a fully penetrant, multifocal breast cancer [129]. By using microarray analysis of unsorted and sorted tumors from this model, as well as NIH3T3 cells transduced with the fusion gene, the authors showed that *ETV6–NTRK3* enriches for WNT target genes through activation of the AP1 complex [129]. The requirement for AP1 activity in *ETV6–NTRK3*-mediated transformation was confirmed by showing that the co-expression of a dominant negative component of AP1 complex, c-JUN TAM67, with the gene fusion blocked tumorigenic properties both *in vitro* and *in vivo* [129]. The *ETV6–NTRK3* gene fusion represents one of the last gene fusions to be discovered by traditional biological techniques.

2.8. TMPRSS2–ETS

In 2005, advances in bioinformatics led to the discovery of rearrangements on chromosome 21 between *TMPRSS2* (transmem-

brane protease, serine 2) and *ERG* (v-ets erythroblastosis virus E26 oncogene homolog (avian)) resulting in the *TMPRSS2–ERG* gene fusion. Thus far, genomic rearrangements leading to an *ERG* gene fusion have been reported in approximately 50% of clinically localized prostate cancers published (reviewed in [130]). *TMPRSS2* is a prostate-specific, androgen-regulated gene [131–133] that has two annotated transcription variants, both of which are involved in the fusion with *ERG*; the annotated *TMPRSS2* in about 50% of the gene fusions, an alternative *TMPRSS2* variant in 10% of gene fusions, and both variants in slightly more than 40% of analyzed gene fusions [134]. *ERG* belongs to the ETS family of transcription factors and has two transcription variants that differ only slightly in the 5'-UTR (deleted in the gene fusion) and in the usage of an in-frame exon, the role of which remains undefined. The most common *TMPRSS2–ERG* gene fusion variants involve *TMPRSS2* exon 1 or 2 fused to *ERG* exon 2, 3, 4, or 5 [134–143] and less frequently rearrangements of *TMPRSS2* exon 4 or 5 fused to *ERG* exon 4 or 5 [141]. In line with the combinatorial complexity of *TMPRSS2–ERG* rearrangements, different fusions have correlated with slightly different phenotypic outcomes. For example, *TMPRSS2* exon 2 fused with *ERG* exon 4 is associated with aggressive disease, while others have been associated with seminal vesicle invasion and poor outcome [143].

Like *TMPRSS2*, the *TMPRSS2–ERG* gene fusion is androgen-regulated in an androgen-responsive cell line (VCAP) carrying the rearrangement [135], but not in an androgen-insensitive cell line harboring the fusion (NCI-H660) [144]. We have shown that VCAP cells and benign prostate cells forced to overexpress *ERG* drive components of the plasminogen activation pathway to mediate cellular invasion using transwell migration assays [145]. We have also reported that primary or immortalized benign prostate epithelial cells overexpressing *ERG* have a transcriptional program with high levels of several invasion-associated genes, but did not display phenotypic increases in cellular proliferation or anchorage-independent growth [145]. Despite this, one group recently identified c-MYC as a downstream target of *ERG* and demonstrated that *ERG* knockdown in *TMPRSS2–ERG* expressing CaP cells resulted in loss of cell growth *in vitro* and loss of tumorigenicity *in vivo*, with only 22% (2/9) mice developing detectable tumors at day 42 in siRNA treated cells as compared to 100% (5/5) in the control group [146]. Interestingly, transgenic mice expressing an androgen-regulated *ERG* fusion gene develop mouse prostatic intraepithelial neoplasia (PIN), a precursor lesion of prostate cancer, not prostate cancer. Taken together with our *in vitro* data, these results suggest that, without secondary molecular lesions such as loss of the tumor suppressors *PTEN* or *NKX3-1*, the *TMPRSS2–ERG* gene fusion may not be sufficient for transformation [145,147,148].

Although *ERG* clearly participates in the majority of ETS family gene fusions in prostate cancer, other ETS family members including *ETV1* [135], *ETV4* [149,150] and *ETV5* [151] also contribute to gene fusions in prostate cancer, albeit at a much lower frequency. In contrast to *TMPRSS2*, which is the only known 5' partner to *ERG*, the other ETS family members may have a variety of 5' partners including those with androgen-responsive promoters (*TMPRSS2*, *SLC45A3*, *KLK2*, *HERV-K_22q11.23* and *CANT1*), one with an androgen-insensitive promoter, but a constitutively active promoter (*HNRPA2B1*), and one with an androgen-repressed promoter (*C15orf21*) [135,149,151–153]. As in the case of *ERG*, forced expression of *ETV1* under the control of a CMV promoter did not enhance cell proliferation in benign prostate epithelial cell lines and did not lead to anchorage-independent colony formation in soft agar, but did lead to the enrichment of genes associated with invasion [145]. Consequently, knockdown of *ETV1* in LNCAP cells prevented transwell invasion through matrigel [145,154].

2.9. EML4–ALK

Recently, Soda et al. reported a retroviral-mediated transformation screen, in which they created a cDNA expression library from a

surgically resected lung adenocarcinoma [155]. Following transformation of NIH3T3 cells, cDNAs were recovered from cells by PCR amplification and sequenced. One of these sequenced transcripts contained a fusion between *EML4* (echinoderm microtubule-associated protein-like 4) and *ALK* (anaplastic lymphoma kinase) that was later confirmed as an inversion of chromosome 2p in 6.7% (5 of 75) NSCLC patients [155]. Wild type *EML4* is a member of the EMAP family of proteins and the amino-terminus (amino acids 1–249) were previously demonstrated to be essential for microtubule formation in HeLa cells [156]. *ALK* encodes a tyrosine kinase and a MAM domain (a domain frequently found on the extracellular side of the membrane on many receptors). Despite the apparent low frequency *EML4*–*ALK* gene fusions in NSCLC, the transforming ability of *EML4*–*ALK* gene fusion variant 1, 2, and 3b, but not a kinase inactive mutant (K589M) has been demonstrated by engrafting NIH-3T3 cells infected with retroviral expression vectors and showing that tumors arise in 8/8 mice from all groups except for the kinase dead mutant [157].

To corroborate the low frequency *EML4*–*ALK* rearrangements in NSCLC, careful PCR-based analysis was completed on NSCLC cases to identify novel in-frame *EML4*–*ALK* gene fusions that led to the identification of two novel fusion isoforms called variant 3a and 3b [157]. Even more recently, analysis of a cohort of 253 lung adenocarcinoma patient samples identified two new *EML4*–*ALK* fusions in which either exon 14 or exon 2 of *EML4* was fused to Exon 20 of *ALK* (variants 4 and 5, respectively), however, only 4.35% of patients were found to express any of the 5 known *EML4*–*ALK* genomic rearrangements [158]. A similarly low rate of the *ELM4*–*ALK* fusion was reported in a study of 104 lung cancer surgical specimens with only one fusion-positive case [159] and, in a study of different lung cancers, the fusion was identified in 3.4% (5 of 149) adenocarcinomas, but not in 48 squamous cell carcinomas, 3 large-cell neuroendocrine carcinomas, or 21 small-cell carcinomas [160]. However, this is to be expected, given the small sample size from non-adenocarcinomas. The *ALK* gene has previously been identified as the 3' fusion partner of *NPM*- [161], *TPM3*- [162], *CLTC*- [163], *ATIC*- [164–166] and *TFG*- [167]. In light of this observation, RT-PCR analysis was used to screen all known hematologic *ALK* fusion partners in a cohort of 77 NSCLC samples, however, no redundant fusion partners were identified and only 2.6% (2 of 77) of NSCLC cases harbored the *EML4*–*ALK* fusion [168]. To supplement the existing RT-PCR data in the literature, our group developed a break-apart FISH assay to analyze *ELM4*–*ALK* fusion as well as the amplification of each gene. We reported the fusion occurred in less than 3% of NSCLC cases analyzed, and that, in most cases harboring the lesion, not all cells exhibited the fusion. We also found that *EML4* and/or *ALK* amplification occurred, indicating that other mechanisms of genomic rearrangement leading to amplification may arise [169].

2.10. *SLC34A2*–*ROS*

In 2007, a survey of phosphotyrosine signaling in lung cancer not only led to the re-identification of the *EML4*–*ALK* fusion, but also the discovery of a novel translocation between chromosomes 4p15 and 6q22, in which the transmembrane domain containing N-terminal region of the solute carrier family 32, member 2 (*SLC34A2*) is fused to an N-terminal transmembrane domain of the c-ros oncogenes 1 (*ROS*), respectively, in the lung cell line HCC78 [170]. *SLC34A2* is encoded from a single transcription variant and *ROS*, which is a type I integral membrane-bound tyrosine kinase and is a known oncogene that is highly expressed in several tumor cell lines, and also encoded from a single transcript. Interestingly, while the authors did not identify *SLC34A2* rearrangements with *ROS* in patient samples, a gene fusion between *CD74*, located at 5q32, and *ROS* was observed, in which the tandem transmembrane domain structure was again observed [170]. This suggests not only that *ROS* is another promiscuous gene fusion partner, but the tandem transmembrane structure is

one mechanism leading to constitutive activation of the tyrosine kinase. Indeed, forced expression of the *SLC34A2*–*ROS* chimera demonstrated constitutive kinase activity in the cellular membrane fraction [170].

2.11. *SLC45A3*–*ELK4*

With the recent advent of next generation sequencing technology (described below), our group has recently identified another recurrent gene fusion in prostate cancer [171]. Using this technology we identified the fusion of *SLC45A3* to *ELK4*, an ETS family member. Here exon 4 of *SLC45A3* is fused to exon 1 of *ELK4*. Interestingly, this novel gene fusion was identified from the RNA of a cell line harboring a known gene fusion involving another ETS family member gene, *ETV1*. Likewise this novel gene fusion involves *SLC45A3*, which is known to fuse with *ETV1* in other prostate cancer cases. Unlike other gene fusions described to this point, *SLC45A3*–*ELK4* seems to result from polymerase read-through and intergenic splicing rather than genomic rearrangement as no detectable alterations were detected on the DNA level by fluorescence in situ hybridization (FISH), array comparative hybridization (aCGH) or high-density single nucleotide polymorphism (SNP) arrays [171]. RNA level gene fusions were recently identified in endometrial stromal tumors and are discussed below.

3. Lessons from MLL translocations

While the list of epithelial derived gene fusions continues to expand, it is important to highlight unique mechanisms of oncogene formation through specific genomic rearrangements from the hematological malignancies. Translocations altering the mixed-lineage leukemia (*MLL*) gene on 11q23 frequently lead to fusions with over 40 different genes on different chromosomes with *MLL*–*AF4* and *MLL*–*AF9* among the most frequent chimeras (reviewed in [172,173]). Interestingly, different *MLL* fusions are highly associated with either acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL), depending on the fusion partner [174]. *MLL* is the mammalian homologue of a *Drosophila* gene called *trithorax*, which was shown to play a critical role in axial morphogenesis and patterning during embryogenesis through the regulation of *HOX* genes (*HOM-C* in *Drosophila*) [175,176]. Multiple studies have suggested that deregulation of *HOX* gene expression contributes to leukemogenesis [177]. Additionally, retroviral transduction of a *MLL* fusion gene construct was able to transform wild type, but not the *Hoxa9*-deficient, bone marrow cells providing direct evidence that specific *HOX* gene expression may be required for leukemogenesis [178]. Because *MLL* chimeras often lose large fragments and different domains from either the N- or C-terminal regions, the seemingly critical role of *MLL*-associated *HOX* gene expression to leukemogenesis led to the question of whether the molecular mechanisms by which wild type *MLL* regulates gene expression are mutually exclusive from those employed by *MLL* chimeras [179].

As the molecular mechanisms of *MLL* target gene regulation continue to unravel, several studies have shed light on the fact that molecular function between wild type and fusion gene settings may be unique, though the outcome of gene activity is ultimately similar. Wild type *MLL* encodes a multi-domain protein with three AT-hooks used for binding AT-rich DNA sequences and a histone methyltransferase domain [180] and assembles into supercomplexes containing several different chromatin remodeling enzymes on target DNA motifs like those found in *HOX* genes [181]. Chimeric *MLL* proteins, on the other hand, appear to utilize different mechanisms to modulate *HOX* gene expression and initiate leukemogenesis. For example, fusion of coiled-coil domains from *GAS7* or *AF1p* to *MLL* endow the chimeric protein with the ability to dimerize on the target gene promoters and have been suggested to stimulate transcription through the inappropriate recruitment of members of the *MLL* supercomplex [182].

This suggested that preventing dimerization of the coiled-coil domains with targeted small molecules could inhibit MLL activity in this subset of MLL fusions. In contrast, some MLL fusions lead to constitutive nuclear retention while maintaining similar binding patterns as the dimerizable MLL chimeras on the HoxA9 locus [183]. In the absence of a partner gene, MLL can acquire an in-frame partial tandem duplication (PTD) of exons 5 through 11 (occurring in approximately 4%–7% of AML cases) that causes overexpression of HoxA7, HoxA9, and HoxA10 in spleen, BM, and blood in a knockin mouse model [184]. As such, altering downstream *HOX* gene expression appears to be one critical role of MLL gene fusions and rearrangements.

Given that wild type and chimeric MLL proteins appear to accomplish at least one similar molecular function (*HOX* gene regulation), the question of how epithelial gene fusions will function in comparison to their wild type counterparts remains intriguing. For example, we have very little understanding of the normal molecular mechanisms utilized by ERG and ETV1 to control gene expression (prostate cancer gene fusions, discussed above), let alone the critical co-factors required for transcriptional regulation. Although we may expect the molecular mechanisms of ERG and ETV1 mediated gene regulation to be the same in the wild type and fusion settings (because the encoded proteins are nearly identical), this remains to be proven. Perhaps the ability to design rational drug targets against specific fusion proteins without obvious molecular susceptibilities (like the tyrosine kinase activity of BCR-ABL) will depend as much on our understanding of each fusion protein's function and critical co-factors as on their downstream targets.

4. Difficulty in identifying epithelial cancer gene fusions

With the discovery of the *TMPRSS2-ERG* gene fusion in prostate cancer, we look back on the history of cancer biology and wonder why gene fusions have not been identified in some of the most well-studied epithelial cancers? Part of the problem was methodological, as the chromosome quality in epithelial neoplasms is very poor when compared to hematologic neoplasms. However, cytogenetic techniques have improved dramatically since the discovery of the “minute” chromosome in 1960 [6]. In fact, in the 1960s, chromosome patterns in epithelial tumors were already being described as abnormal [185] and it was often thought that the degree of cytogenetic changes corresponded proportionally with clinical progression [186], making the identification of individual and recurrent translocations difficult. In fact, the idea that the induction of genomic instability is a critical and intended step in the malignant progression of solid tumors has gained considerable momentum [187,188]. Recently, it was demonstrated that overexpression of Separase, a protein that is overexpressed in a subset of breast cancers, leads to can induce chromosome instability and aneuploidy in the mutant p53 mouse mammary epithelial cell line FSK3 [189]. Likewise, deregulation of Mad2, which regulates separase activity, has been shown to promote chromosomal instability, induce aneuploidy and lead to tumorigenesis [190]. Interestingly, once Mad2-induced neoplastic transformation has occurred, Sotillo et al. demonstrated that expression of Mad2 is no longer required for tumor progression suggesting that the induction of chromosomal instability could be a transient event in oncogenesis [190]. In fact, it is possible that specific gene fusions induce genomic instability through deregulation of normal mitotic events like separase or Mad2 activity or through novel mechanisms yet to be described. If induction of chromosomal instability was a mechanism of oncogenesis employed by a specific gene fusion, then induction of other secondary “carrier” chromosomal rearrangements would simply serve to mask the identification of the recurrent genetic rearrangement. Such a progression pattern in epithelial tumors could explain the complex heterogeneity often observed in such malignancies (Fig. 5). In contrast, leukemias, lymphomas and mesenchymal tumors

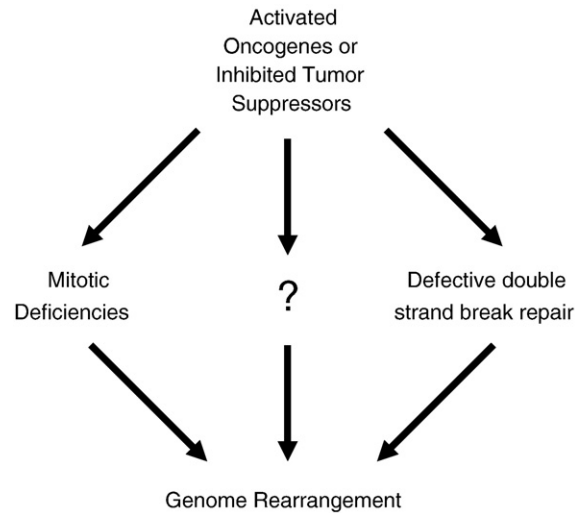


Fig. 5. Difficulty in discovering gene fusions. One possibility is that a critical function of oncogenes in epithelial cancers is to alter genomic structure and it has been suggested that such changes could lead to cancer progression. However, if such a model were true, it would give a reason for the genomic heterogeneity observed in epithelial cancers that has allowed recurrent gene fusions to go unnoticed in solid tumors.

are almost 95% clonal [191]. As such, the complexity and sheer number of genomic rearrangements in epithelial malignancies has led to difficulty in defining primary aberrations in these neoplasms. This difficulty eventually led to the incorrect notion that genomic rearrangements leading to gene fusions were simply less common in epithelial tumors.

5. Mitelman hypothesis

In order to address this notion that fusion genes are almost exclusively a hematologic phenomena, Mitelman et al. completed a comprehensive study of all known cytogenetically abnormal neoplasms reported in the literature [192]. Importantly, data published by the group supported the counter-hypothesis that, in every tumor type, the numbers of recurrent balanced chromosome abnormalities, gene fusions and balanced rearrangements are a function of the total number of analyzed cases [192]. In this study, 271 gene fusions and 59 potential gene fusions (only one gene identified at the break-point) were catalogued, of which 275 unique genes were involved in the rearrangements [192]. This indicated that a substantial number of genes were present in more than one chimeric transcript (e.g., *MLL*, *ETV6* and *RET* as described above). In classifying each gene fusion by the class to which each member of the chimera belonged, the group demonstrated that the proportion of fusions belonging to each class was approximately equal in both hematologic and solid tumor malignancies, with the transcription factor class accounting for 38–44% and tyrosine kinase class tabulating 5–7% [192]. This study suggested that the occurrence of gene fusions is a general molecular event that has no fundamental tissue-specific differences. However, gene rearrangements must at least encourage function in specific genetic backgrounds such as the *TMPRSS2-ERG* fusion, which requires active androgen signaling, and thus encourages prostate specificity.

6. Tissue-specific gene fusions

The idea that genomic rearrangements are tissue-specific is an emerging concept in the field of gene fusion biology. For example, *TMPRSS2* is a strongly androgen-regulated and prostate-specific gene that is fused to the ETS family members ERG and ETV1 in prostate cancer [135]. While other ETS family members form fusion genes

Table 1
Chromosomal rearrangements in epithelial cancers.

Malignancy	Gene fusion	Chromosome rearrangement	Method of discovery	Study	Ref.
Follicular thyroid carcinoma	PAX8–PPAR γ	t(2;3)(q13;p25)	Primary tumor karyotypic analysis/FISH/3' RACE	Kroll et al.	[90]
Midline carcinoma	BRD3–NUT	t(9;15)(q34;q14)	Candidate gene FISH Screen	French et al.	[106]
	BRD4–NUT	t(15;19)(q14;p13)	Primary tumor karyotypic analysis/FISH/southern blot	French et al.	[98]
Non-small-cell lung cancer	EML4–ALK	inv(2p)	Transformation assay/direct sequencing	Soda et al.	[155]
	TFG–ALK	t(2;3)(p23;q12)	Tyrosine Kinase Activity Screen/5' RACE	Rikova et al.	[170]
	SLC34A2–ROS	t(4;6)(p15;q22)			
Papillary renal cell carcinoma	PRCC–TFE3	t(X;1)(p11;q23)	Primary tumor karyotypic analysis/southern blot/5' RACE	Sidhar et al.	[69]
Papillary thyroid carcinoma	RET–NTRK1	t(1;10)(q21;q11)	Transformation assay/direct sequencing	Martin-Zanca et al.	[24]
Pleomorphic adenoma	CTTNB1–PLAG1	t(3;8)(p21;q12)	Primary tumor karyotypic analysis/ Breakpoint mapping/southern blot/5' RACE	Kas et al.	[59]
	HMGA2–FHT	t(3;12)(p14;q15)	Primary tumor karyotypic analysis/3' RACE	Geurts et al.	[80]
	HMGA2–NFIB	t(9;12)(q24;q15)	Primary tumor karyotypic analysis/3' RACE	Geurts et al.	[81]
Prostate cancer	TMPRSS2–ERG	del(21)(q22)	COPA/Exon walking/5' RACE	Tomlins et al.	[135]
	TMPRSS2–ETV1	t(7;21)(p21;q22)			
	TMPRSS2–ETV4	t(17;21)(q21;q22)		Tomlins et al.	[149]
	TMPRSS2–ETV5	t(3;21)(p28;q22)		Helgeson et al.	[151]
	SLC45A3–ELK4	del(1)(q32)	Integrated high-throughput sequencing	Maher et al.	[171]
	DDX5–ETV4	t(17)(q24;q21)	Candidate gene FISH Screen/5' RACE	Han et al.	[150]
Secretory breast carcinoma	ETV6–NTRK3	t(12;15)(q13;q25)	Primary tumor karyotypic analysis/FISH	Tognon et al.	[108]

that give rise to other malignancies, chimeras between androgen-regulated genes and ETS genes have only been observed in prostate cancer [130]. Likewise, the ALK tyrosine kinase is frequently fused to multiple partners in hematopoietic (myelogenous leukemia), mesenchymal (congenital fibrosarcoma) and epithelial (secretory breast carcinoma) malignancies, but no redundant fusion partners have been identified across tissue types [159]. Retention of the TFE3 DNA binding domain in follicular thyroid carcinoma is another example of this, as TFE3 is a thyroid-specific transcription factor [93]. Importantly, little is understood about the molecular mechanisms leading to gene rearrangement and the underlying reasons that particular chimeras are formed recurrently. The idea that tissue-specific rearrangements occur by fusing highly transcribed genes holds promise and would at least partially explain the apparent tissue specificity observed in the formation of chimeric transcripts even between genes that are fused in multiple cancer types.

The idea that gene fusions are tissue-specific could have profound implications on the discovery of novel gene fusions. Clearly, however, gene fusions do not always confer tissue specificity. HMGA2 has a 3'-UTR that is negatively regulated by the Let7 microRNA and simply replaces its 3'-UTR through rearrangement with another gene (described above), therefore representing a gene fusion that most likely retains functionality in multiple tissue types. As such, while this concept may have its largest impact on underlying molecular mechanisms of newly discovered gene fusions, it will probably not alter the rate gene fusion discovery.

7. Discovery of novel gene fusions

Although the rate recurrent chromosomal rearrangement discovery in epithelial tumors has been modest, the recent discovery of gene fusions in prostate cancer has led to a renewed interest in gene fusions identification in other epithelial cancer subtypes. Perhaps the best explanation for the sudden increase in the characterization of recurrent gene fusions is the advent of novel technologies (Table 1). For example, the use of existing gene-expression data in the discovery of novel gene fusions was limited until the emergence of cancer outlier profile analysis (COPA), which ranks genes by normalizing expression values based on median absolute deviation of gene expression to accentuate outlier profiles (reviewed in [130]). When COPA was applied to gene-expression datasets in the Oncomine database [193–196], the analysis was able to identify several hallmark cancer related genes and led to the discovery of the ERG and ETV1 outlier profiles in prostate cancer [135]. Subsequent exon-walking quantitative PCR was used to demonstrate loss of the 5' exons in both

ERG and ETV1, giving rise to the notion that a gene fusion event was responsible for the outlier expression of these genes in prostate cancer. Finally, 5'-RNA ligase-mediated rapid amplification of cDNA ends (5'-RACE) was used to identify the 5' untranslated region of TMPRSS2, a prostate-specific, androgen-regulated, transmembrane serine protease gene [131,132,197]. Fusion specific PCR and fluorescence in situ hybridization (FISH) were used to confirm the genomic rearrangement.

In contrast to using COPA and exon-walking quantitative PCR to identify fusion gene candidates, several labs are now employing next generation sequence methods wherein DNA or mRNA can be fragmented, sequenced and mapped to the genome in a matter of weeks to identify gene fusions. Various commercial platforms have been developed with the intent of sequencing as much of the genome or transcriptome as possible and are classified based on the length of the templates each platform sequences. Long read technologies, like 454, can sequence long templates (>1 kb) whereas short read technologies, like SOLEXA and SOLID, are currently capable of sequencing 35–50 nucleotide templates. At first glance, long read technologies may appear to have the advantage of making genome (or transcriptome) re-assembly much simpler than short read technologies. However, a major advantage of short read technologies is the depth of coverage, or the number of times a segment of the genome is sequenced, which is currently much higher for short read than long read technologies. As such, the choice of technology is still dependent on the scientific question.

If our question is to identify the best method for novel fusion gene discovery, we assume that sequencing the transcriptome space will be much efficient than sequencing cancer genomes. In theory, the discovery of gene fusions by long read technology will require sequencing across the actual gene fusion boundary of the chimeric transcript. In contrast, short read technologies may be able to identify gene fusions by two different methods. The first and most straight forward method is the identification of sufficient short reads that do not map directly to the transcriptome, but correspond to the gene fusion boundary; and these short reads should identify both contributing genes with high probability. Second, because transcripts are thought to be sequenced with a uniform distribution across the length of the transcript, except for at the extreme 5' and 3' ends, exon expression for each transcript can be analyzed. Genes involved in rearrangements, leading to chimeric transcripts, would be expected to lack any exon expression on one of the transcript ends. However, this method will need to be carefully developed, as mapping of short reads to duplicated sequences (or sequences that appear more than one time in the genome) remains challenging.

To test whether short or long read technology was better for the discovery of recurrent gene fusions, we recently sought to “rediscover” the known gene fusions BCR–ABL1 and TMPRSS2–ERG by sequencing the RNA transcriptome of either the leukemia cell line K562 or the prostate cell line VCAP, respectively, with both short and long read platforms [171]. Initially both technologies were able to identify the known gene fusion from the sample, but were also able to identify several other candidate gene fusions. For example, the Illumina short read platform nominated 428 candidates from the VCAP cell line [171]. However, most of these candidates were likely to result from either trans-splicing [198], co-transcription of adjacent genes followed by intergenic splicing [199], or as a consequence of the sample preparation protocol. In order to reduce the list of potential candidate genes, we intersected the results of the two platforms to yield a much more condensed list. Indeed by integrating the short read and long read platforms rather than constraining the analysis to either short or long read technology, we were able to significantly reduce the percent of false positive gene fusions discovered [171].

In the future, an even newer adaptation of next generation sequencing will likely replace the current reliance on both short and long read technologies for fusion gene discovery. Paired end sequencing is a method in which short read technology is used to sequence nucleotides from both the 5′ and 3′ ends of 200–300 nucleotide fragments of the genome (or transcriptome). By sequencing both ends of a fragmented RNA, paired end sequencing enhances not only the reliability of mapping and assembly, but also maintains significant sequencing depth. In a manner similar to our recent integration of short and long read platforms, the use of paired end sequencing technology for gene fusion discovery should first be examined by comparing the ability of matched mate-pairs to identify known gene fusions from control samples. With paired end sequencing, a single sample preparation and individual sequencing run will hopefully provide sufficient coverage for gene fusion discovery and these improvements as well as other advancements in modern sequencing technologies will likewise lead to a dramatic improvement in our ability to identify novel, pathogenic gene fusions.

8. Lessons from the JAZF1–JJAZ1 chimera

Advances in sequencing technology will most likely lead to a rapid increase in the number of characterized gene fusions over the next few years. However, a much more pertinent question may address the reasons for chromosomal rearrangements leading to gene fusions. Could fusion transcripts be a part of normal cell biology? It is also plausible that tissue-specific fusions could impart growth advantages that allow a cell to survive traumatic stress. Nonetheless, while the underlying molecular mechanisms triggering genomic rearrangement are still unclear; we surmise that once a genomic rearrangement occurs, cells harboring favorable gene fusions will be selected over time.

Insight into the development of genomic rearrangements may come from fundamental observations made following the study of endometrial stromal (EMS) tumors. In 2001, a recurrent translocation t(7;17)(p15;q21) was demonstrated to occur in EMS tumors that led to expression of the chimeric JAZF1/JJAZ1 mRNA transcript [200]. Although the mechanism leading to this rearrangement remains unknown, a recent study demonstrated that trans-splicing of RNAs in normal human endometrial stromal cells can lead to the chimeric JAZF1/JJAZ1 RNA and protein independent of chromosomal rearrangement [201]. This observation suggests that certain gene fusions may be generated by trans-splicing of RNAs, which then lead to chromosomal rearrangement due to their pro-neoplastic nature. Interestingly, the group also demonstrated that the RNA trans-splicing event leading to the JAZF1/JJAZ1 chimera was inhibited at high concentrations of either estrogen or progesterone, further suggesting that certain RNA fusions may occur in a hormone-dependent manner.

The question of whether or not other specific gene fusions arise due to abnormal exposure to specific hormones has not been studied.

9. Conclusions

A limited number of epithelial gene fusions have been described and the quest for novel recurrent gene fusions, like the discovery of TMPRSS2–ERG gene fusions in prostate cancer, may provide major advances in cancer research in the near future. Here, we have demonstrated that gene fusions lead to overexpression or constitutive activation of oncogenes by a variety of unique mechanisms including fusion of housekeeping or tissue-specific gene promoters to oncogenes, as in the case of TMPRSS2 gene promoter and 5′-UTR to ERG or, as in the case of HMGA2, through evasion of a microRNA by replacement of an oncogene’s 3′-UTR. Despite the multitude of mechanisms used by chimeric transcripts to drive malignancy, several important lessons can be taken from characterized epithelial gene fusions, studies of MLL translocations, as well as the very recent discovery of JAZF1–JJAZ1 RNA fusions, which precede genomic rearrangement in specific cell types.

As in the case of Imatinib and BCR–ABL1, perhaps the one of the best methods for interfering with the development of specific malignancies will be through inhibition of well-characterized, pathogenic fusion genes with rationally designed molecularly tailored therapies. In the future, the use of both COPA and high-throughput massively parallel sequencing will greatly increase the speed and reliability of fusion gene discovery on both the genomic and transcriptomic levels. We expect many more gene fusions to be reported over the next several years in various tumor types, many of which will hopefully serve as rational drug targets.

Acknowledgements

We thank Jill Granger for critically reading the manuscript. A.M.C. is supported by a Burroughs Wellcome Foundation Award in Clinical Translational Research. This work was supported in part by the National Institutes of Health Prostate SPORE P50CA69568 to A.M.C., and the Early Detection Research Network (U01 CA113913 to A.M.C.) and the Department of Defense (PC051081 to A.M.C., BC083217 to J.C.B.).

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RhoC Expression and Head and Neck Cancer Metastasis

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Key words: Tumor metastasis, lentiviral transduction, CD31, lung metastasis, RhoC

Running Title: RhoC is essential for head and neck metastasis

Total number of pages: 23 (excluding title page); total number of figures: 6.

Grant and financial support: National Cancer Institute Head and Neck Cancer SPORE grants (TN Teknos and SD Merajver)

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Abstract

In this study, we have investigated the correlation between RhoC expression and the metastatic behavior of head and neck squamous cell carcinoma. The inhibition of RhoC expression was achieved using small hairpin RNA (shRNA) and lentiviral transfection and transduction technology. Fluorescence microscopy of the RhoC knockdown stable clones showed a strong green fluorescence in the majority of cells, signifying a high efficiency of transduction. qRT-PCR of lentivirus infected cell lines showed a 70-80% reduction in RhoC mRNA expression. Furthermore, the mRNA expression levels of other members of the Ras superfamily did not show any significant decrease. Cell motility and invasion were also markedly diminished in RhoC depleted cell lines as compared to parental lines. Hematoxylin and eosin staining of lung tissue obtained from the lungs of SCID mice implanted with RhoC knockdown cell lines showed marked decrease in lung metastasis and inflammation of the blood vessels. When cultured, lung tissue showed a significant decrease in cell growth in the mice which were implanted with RhoC depleted cell lines as compared to either parental or shRNA scrambled sequence control lines. Microscopic studies of CD31 revealed substantial quantitative and qualitative differences in the primary tumor microvessel density as compared to its parental and shRNA-scrambled control. This study is the first of its kind to establish the involvement of RhoC in head and neck metastasis. These findings suggest that RhoC may be a novel target for biologic therapeutic targeting in the future.

Introduction

Head and neck cancer is the sixth most common cancer world wide (1). As per the statistical report of the American Cancer Society, about 40,000 new head and neck squamous cell carcinoma are diagnosed every year in the United States. Among these, most of the patients are diagnosed at a very late stage (stage III and IV). Despite the advancement in surgical procedures, chemo and radiation therapy survival rates have not improved in the last several decades (2). Furthermore, it has been shown that the high rate of morbidity is due to both locoregional recurrence and distant metastases.

In the past decade, numerous studies have shown that the Rho family of GTPases, (RhoA, RhoB, RhoC, Rac1, Rac2, Rac3 and Cdc42) are involved in malignant progression towards metastasis. In fact, several studies have also reported elevated RhoA

and RhoC expression in a number of tumor types (3, 4). Among the Rho protein family, RhoC (molecular-mass of approximately 21 kDa) has been implicated in a wide range of cellular activities, including gene expression, cell proliferation, intracellular signaling, and cytoskeletal organization (5). More significantly, RhoC plays a central role in the assembling and facilitating actin-myosin contractions that enhance focal adhesion, resulting into cell polarity, increased cell motility and, consequently, increased invasiveness (6-8). In addition, signaling mediated by Rho proteins through Rho activating kinase (ROCK) regulate proteins that in turn regulate actin polymerization such as cofilin, profilin and Formin homology (FH) proteins (9). Interestingly, high levels of RhoC and ROCK are also associated with membrane blebbing, a phenomenon that is observed in motile or invasive cells (9, 10).

RhoC over-expression is now well documented in a wide range of malignant cancers suggesting an important role in changing non-invasive carcinomas into invasive forms. Interestingly, over-expression of RhoC has been reported in inflammatory breast cancer and exclusively in invasive breast carcinoma (11-14). Other tumor types where over-expression of RhoC has been reported are ovarian carcinoma (15), esophageal squamous cell carcinoma (16), pancreatic cancer (17), gastric cancer (16, 18) and human melanoma (10, 19). In addition, functional studies have shown that RhoC can act as a transforming oncogene when it is over-expressed in human mammary epithelia converting these normally immobile cells into highly motile and invasive malignant cells (14, 20). Thus, a wide range of current studies reveal an important role of RhoC in cancer metastasis.

However, very few studies to date have investigated the role of RhoC in head and neck cancer. Studies on gene expression profiling of stage III and IV regionally metastatic head and neck squamous cell carcinoma (HNSCC) showed that there is elevated levels of RhoC when compared to stage I and II localized malignancy (21). Furthermore, studies in our laboratory have shown that there is elevated RhoC expression in tumors of patients with HNSCC when compared to normal squamous cell epithelium (20). More importantly, our study showed that increased RhoC expression is strongly associated with lymph node metastasis and could also be used to predict metastasis even in small (T1,T2) primary tumors (22). In the present study, we investigated the role RhoC in head and neck metastasis by inhibiting its function using RNA interference (RNAi). Our *in vitro* findings determined that inhibiting RhoC function strongly reduced cell motility and invasion. Furthermore, we observed a remarkable reduction in tumor metastasis and microvessel density in SCID mice injected with RhoC knockdown cell lines. These findings suggest that inhibition of RhoC function in head and neck squamous cell carcinoma can diminish this tumors' aggressive behavior thus opening new possibilities for future drug therapies targeting this pathway.

Materials and Methods

Cell culture

University of Michigan squamous cell carcinoma cell lines (UM-SCC)-11A and (UM-SCC)-1 are a well established cell lines derived from a 65-year-old patient with a T2 N2a of the epiglottis and 46-year-old patient with T2N0 of the false vocal cord (23, 24). These cell lines were grown at 37°C in a humidified atmosphere with 95% air-5%

CO₂. The cultures were maintained in Dulbecco Modified Eagle Medium (DMEM), (Gibco/BRL; Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum (Hyclone; Logan, UT) and supplemented with 50µg/ml of penicillin G, and 50µg/ml of streptomycin sulphate. Sub-culturing of 80% to 90% confluent cells was routinely performed using trypsin-EDTA solution (0.05% trypsin and 0.53mM EDTA). At harvest, the cells were treated with trypsin, washed, concentrated by centrifugation, and counted with a hemocytometer. The cells were assessed for viability by typan blue exclusion test (>95%) and then re-suspended to a final density of 5.0×10^6 cells per ml DMEM.

Lentivirus infection and selection of positive RhoC knockdown clones

RhoC knockdown and scrambled sequence constructs with green fluorescence protein (GFP) tag and puromycin resistance sites were synthesized by the vector core facility of the University of Michigan (www.med.umich.edu/vcore). The sequences used for RhoC constructs are available in open biosystems (www.openbiosystems.com) and are: oligo ID V2LHS_69446 and V2LHS_69410, accession number NM_001042678. The sequences of the constructs are 69446 = 5'-ATACTGTCTTTGAGAACTATAT (Sense) [for RhoC knockdown clone 1] and 69410 = 5'-CACCAGCACTTTATACACTTC (Sense) [for RhoC knockdown clone2]. The sequence of shRNA miR non-silencing (scrambled) control: ATCTCGCTTGGGCGAGAGTAAGTGCTGTTGACAGTAAGC GATCTCGCTTGGGCGAGAGTAAGTAGTGAAGCCACAGATGTACTTACTCTCG CCCAGCGAGAGTGCCTACTGCCTCGGA. This control sequence does not match any known mammalian genes (the sequence had at least 3 or more mismatches against any gene which was determined via nucleotide alignment/BLAST of target 22mer sequence).

This is the non-silencing shRNAmir hairpin sequence found in the pSM2, pSMP, pGIPZ, pTRIPZ and pLemiR non-silencing controls. The entire process of lentivirus infection and selection has been categorized in the following sub headings. (a) ***CaCl₂ transfection of lentiviral packaging 293 FT Cells*** : The 293FT cell line was procured from Invitrogen, Carlsbad, CA. Fresh DMEM-10% FBS with 25µM chloroquine was added to 60-70 % confluent 293FT cells, (which were previously seeded at a cell density of $\sim 5 \times 10^6$), and incubated at 37°C for an hour. In a total 500µL reaction volume, 4µg DNA constructs, 4µg each of viral packaging vectors, namely Gag, Pol, and Env, and 62.5µL of 2M CaCl₂ solution (final concentration 250 mM) were added. This reaction mixture was titrated to 500µL against 2X HBS buffer, pH 7.02. One milliliter of this solution contained viral particles was added onto the 293FT cells. Next, cells were incubated for another 12 hours at 37°C. Media was changed after 12 hours to remove chloroquine and fresh DMEM-10% FBS was added to the growing 293FT cells to produce virus. (b) ***Infection of cell lines with lentivirus***: Supernatant of the 293FT cells was filtered through 0.45µM filter. One milliliter of this filtered DMEM was added to the UM-SCC-11A and -1 growing in 6 well culture plates. Cells were incubated at 37°C and the GFP expression was monitored after 48 hours of infection. (c) ***Selection of positive colonies of cells***: Pre-optimized puromycin (1.6 µg /ml) was used for the selection of the lentivirus infected RhoC knockdown clones. These clones were further sorted by flow cytometry to get maximum number of GFP positive cells, which were used in subsequent studies.

Flow cytometry analyses

About 70-80% confluent lentivirus infected cells were harvested using trypsin-EDTA solution and re-suspended in phosphate buffer saline containing 3% FBS, 0.5mM

EDTA and 60U/ml DNase. Flow cytometry analysis was performed using a BD FACS Aria IIU flow cytometer equipped with a 488nm, 15mW, air-cooled Argon laser. (Analytical Cytometry Laboratory, Ohio State University Comprehensive Cancer Centre). GFP positive cells were sorted out and grown for subsequent experiments.

Quantitative reverse transcriptase polymerase chain reactions (qRT-PCR)

Total RNA was isolated according to the standard procedure using TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative Reverse transcriptase polymerase chain reactions (qRT-PCR) were conducted by Taqman probe system, from Applied Bio Systems (Foster City, CA) by using the following products cd42: Hs03044122_g1, Rac1: Hs01025984_m1, Rac2: Hs01032884_m1 and RhoC: Hs00733980_m1. Beta actin and G3PDH were used as the data normalizers. Relative changes in gene expressions were calculated using $2^{-\Delta\Delta C_T}$ method (25).

Cell Invasion and Motility Assay

Invasion Assay: Cell invasion assays were performed using BD BioCoat Matrigel Invasion Chamber which was obtained from BD Biosciences, Bedford, MA USA. The procedure was followed according to manufacturer instructions. Briefly, about 2.5×10^5 cells in 2 ml of serum free DMEM were added at the top of the insert and 1ml of media was added in the bottom wells of each insert. Fetal bovine serum albumin (FBS) was added in the media of lower chamber (final concentration of FBS was 10%, v/v) which acts as a chemo attractant. Cells were incubated for 22 hours in a humidified cell culture incubator, at 37°C, 5% CO₂ atmosphere. Next, the non-invading cells at the top of the insert were scraped out with the help of cotton-tipped swab. The invading cells which were attached to the under side of the membrane were fixed in 100% methanol and

stained with 1% Toluidine prepared in 100% methanol. After repeated washing of the membrane with distilled water, stained cells were allowed to air dry at room temperature before it was visualized under microscope. A parallel experiment with control insert (without Matrigel) was also run. Matrigel invaded cells were counted microscopically at X 100 magnification.

Motility assay: Cell motility assays were performed in 100mm Petri dishes. At about 80% confluence, cells were washed with PBS and a fine scratch in the form of groove was made by the help of sterile pipette tip and immediately photographed. We designate this time as the zero hour. Next cells were supplemented with DMEM containing 10% FBS and allowed to grow. Migration of cells from the edge of the groove towards the centre was monitored microscopically after 24 hours to assess the extent of scratched area covered. The width of the scratch was measured at zero hour and after 24 hours to calculate the percentage of the gap covered by the cells in 24 hours time.

Animal Model

Athymic severe combined immune deficient (SCID) mice were obtained from the Jackson laboratory, Bar Harbor, ME- USA, 6 weeks old mice were housed in cages of 5 animals in each. Five animals per treatment were selected to receive parental, shRNA-scrambled sequence control and RhoC knockdown clone, resulting 15 animals per cell lines per set of experiments. About half a million UM-SCC-11A and -1 cells were suspended in 100µl serum free DMEM and injected thorough the tail vein and or in the flank region into the mice using 0.5 inch, 27-gauge needle. Animals were monitored every other day for their general health and activities. At the end of second weeks the animals were euthanized using a CO₂ chamber. The lungs were dissected and half of the

lungs were fixed in buffered formalin for 6 hours thereafter transferred to 70% methanol and then processed to form paraffin embedded tissue blocks Hematoxylin and eosin (H and E) staining were done. Remaining half of the lungs was digested in collagenase for culturing the cells. At the end of week 12, tumors in flank region was fully grown. The animals were euthanized and tumors were dissected and fixed in the same way as described above for CD31 staining.

Lung Metastases

Slides of five-micrometer sections of lung were prepared and H & E stained.. Five random fields in a blind fashion way were examined microscopically at low powers (X40 and X100 magnifications) to detect metastases.

Microvessel Density

Microvessel density in all primary tumors was assessed using anti mouse CD31 antibody (PharMingen, San Diego, CA) at a dilution of 1:250. Five random high power fields (X40 and X100 magnifications) were selected to visualize the microvessels. The mean was reported in a blind fashion for each tumor.

Statistical Analysis

Statistical analyses were performed using sigma graph pad prism 4 software. The mean was reported with Standard deviation ($\pm$ SD). Differences were considered to be statistically significant when *p* values were less than 0.05.

Results

RhoC mRNA expression is greatly reduced in knockdown clones from head and neck squamous cell carcinoma cell lines

The inhibition of RhoC function was achieved using small hairpin RNA (shRNA) and the lentiviral transfection and transduction technology. Briefly, the cell lines were transduced with highly modified lentiviral vectors that carry small sense and anti-sense sequences against the RhoC gene. In the infected cells, these sequences are transcribed into small hairpin RNAs (shRNA), which then trigger endogenous and highly specific RNA degradation machinery that targeted RhoC mRNA being synthesized.

To test the lentiviral infection efficiency and for the selection of positive clones (RhoC knockdown) in the selected cell lines, the vector was designed to express Green Fluorescent Protein (GFP) and puromycin resistance gene. After lentiviral infection, positive (stable) clones were selected using Puromycin (1.6 μ g /ml) antibiotic. As shown in figures 1A and 1B flow cytometry revealed that the numbers of non-infected cells were significantly low. In addition, fluorescence microscopy of the stable clones showed a strong green fluorescence in the majority of the cells, signifying a high efficiency of transfection. The GFP positive cells were further sorted out and re-grown for subsequent experiments.

We then tested the effectiveness of shRNA in depleting RhoC mRNA expression by real time quantitative PCR (qPCR) in our selected cell lines. Because only a small number of specific gene sequences are capable of activating the RNA degradation pathway, we used two different RhoC knockdown clones (namely C1 and C2 along with a parental and shRNA-scrambled sequence infected control) to ensure the effectiveness of depleting levels of RhoC. The results show greatly reduced expression levels of RhoC gene in the C1 and C2 RhoC knockdown clones, while normal RhoC expression was observed in clones with shRNA-scrambled sequence (Fig.2). The relative RhoC mRNA

expression in parental, shRNA-scrambled control and RhoC knockdown clones 1 and 2 were evaluated by quantitative RT-PCR and the C_T values thus obtained were normalized using two house keeping genes as described in material and methods sections. As shown in figure 2A, mRNA expression decreased about 75 % and 80 % in RhoC knockdown clone 1 and clone 2 of UM-SCC- 1, respectively, while a decrease of 40% in clone1 and 70 % in clone 2 of UM-SCC-11A was observed. The control shRNA-scrambled sequence in either of the cell lines did not show any significant reduction in RhoC mRNA expression level. To confirm that only RhoC expression was being inhibited, the mRNA levels of other Rho family members, Cdc42, Rac1 and Rac2 were analyzed by quantitative RT-PCR. As shown in figures 2B, C and D, the expression levels of, Cdc42, Rac1 and Rac2 are not affected significantly, thus confirming that our shRNA process is highly specific to RhoC only. These studies provided a clear insight about the “switching off” of the RhoC machinery by decreasing total levels of RhoC mRNA expression, and, therefore, further detailed studies on its functional roles are defensible. One of the most basic clinical questions that arise at this point is how knockdown of the RhoC transcript affects metastasis in head and neck cancer. To address this question, we investigated two characteristic behaviors of metastatic cells, invasion and motility, in the transduced cell lines.

RhoC knockdown clones show decrease in cell invasion and motility

Invasion assay: In our study we found that RhoC-depleted clones of UM-SCC-11A and -1 were remarkably less invasive and motile than their parental or shRNA-scrambled controls (Figs. 3A and B). Remarkably, cell invasion was decreased by 50% and 75% in RhoC knockdown clone 1, and 2, respectively, of UM-SCC- 11 A and 60%

and 80% in clones 1, 2 of UM-SCC-1 as compared to their parental or shRNA-scrambled controls (n=3; p< 0.003).

Motility assay: As shown in figures 4A and B, a noticeable decrease in cell motility in RhoC knockdown clones clearly indicates its involvement in preventing the metastasis in head and neck patients (n=3; p< 0.005). All these *in vitro* results provide ample supporting evidence that RhoC plays an important role in cell invasion and metastasis in head and neck cancer, while depleted RhoC may be a useful tool for therapeutic measure in head and neck carcinoma.

RhoC plays an important role in lung metastasis and microvessel density formation

Besides localized tumor, lung metastasis is common in head and neck cancer patients (26). Keeping in view this aspect we designed an *in vivo* study where we can analyze the effect of RhoC inhibition in lung metastasis and in primary tumor vascularity. In our *in vitro* study, we found that of RhoC knockdown clones 1 and 2 in the cell lines tested were gave very similar results in our motility and invasion assays. Therefore, for further *in vivo* studies, we selected only clone 2 from both cell lines to test for metastasis. Three groups of SCID mice with five mice in each group were used for *in vivo* studies. Half a million cells from parental, shRNA-scrambled control and RhoC knockdown clone 2 were injected through tail vein and about a million cells were implanted in flank region for further studies. Two weeks later lungs were dissected, half of the lungs were stained with H and E and remaining half were cultured. Figure 5A shows a remarkable difference in lung metastasis between parental, shRNA-scrambled control and RhoC knockdown clone. The bar graph also shows the number of cancer cells grown in digested lung of mice which includes parental, shRNA-scrambled control and RhoC knockdown clones. A

huge lump of metastatic, highly inflamed tissues and blood vessels were found in lung region of the mice injected with either parental or shRNA-scrambled control while few and very small patches can be seen in the lungs of the mice injected with RhoC knockdown clones. A bar graph shows a 67% and 58% decrease in cell number in RhoC knockdown clones when compared to their parental lines (Fig.5B). These results strongly suggest that inhibition of RhoC expression greatly reduces metastasis *in vivo*. Furthermore, microvessel density of the localized solid primary tumor which grows into a sizable volume after 12 weeks of implantation in the flank region was analysed using CD31 antibody. Microscopic analysis of the CD31 stained tumor revealed a remarkable difference in microvessels formation in RhoC knockdown clones which were very small and poorly developed as compared to the corresponding parental or shRNA-scrambled control (figure 6). A similar pattern of lung metastasis was also observed in UM-SCC-11A (data not shown).

Discussion

Tumor metastasis is well correlated with the over-expression of certain oncogenes. The over-expression of the Rho gene family has been reported in many malignant forms of cancer (27), including pancreatic cancer (17), gastric cancer (16, 18) and human melanoma (10, 19). However, there have been very few studies on whether over expression of RhoC is involved in head and neck metastasis. Previous studies in our laboratory have shown that RhoC is actively expressed in several well established University of Michigan Squamous Cell Carcinoma cell lines (UM-SCC). Among the cell lines tested, the UM-SCC- 11A and -1 lines exhibited considerably high levels of RhoC (22). In particular, the active form of RhoC (RhoC GTPase) was observed to be

constitutively expressed in the UM-SCC lines. Therefore, for our current study, we selected two UM-SCC lines (UMSCC-11A and UMSCC-1) to evaluate the role of RhoC in head and neck squamous cell carcinoma metastasis. Our first and foremost aim was to inhibit RhoC expression in the two selected cell lines and analyze its function *in vitro*. Our expectation was that the motility and invasiveness would be greatly reduced in RhoC depleted cell lines as compared to parental lines. The data from the present study shows that the newly emerging tool of gene silencing using lentiviral infection is an efficient way to achieve this goal. The characteristic feature of lentiviral infection is that it can integrate into the genome of not only dividing cells but also non dividing cells. By doing so it achieves stable, long term expression of shRNAs. The inhibition of cervical cancer and melanoma growth by using lentivirus gene silencing strategies has been well established (28, 29). In this study we have demonstrated a successful inhibition of RhoC gene expression and, subsequently, function using shRNA techniques (Fig. 2). Furthermore, our data show that cell invasiveness and motility which are characteristics of aggressive head and neck cancer cell lines were diminished when RhoC expression was inhibited (Figs.3 and 4). Therefore, these results suggest that RhoC over-expression drives cell invasion and motility in HNSCC. It is reported that one of the major functions of Rho-family of proteins is to control cytoskeletal organization (30). Cytoskeletal proteins are involved pre-dominantly in cell motility. Therefore, RhoC may control metastasis by modulating cell motility (31). In order to facilitate the movement of cells, they need to turn over both cell-extra cellular matrix and cell-to-cell adhesions which includes both adherence junctions and tight junctions (32, 33). It has also been reported that RhoC plays a predominant role over RhoA in the weakening of adherence junctions,

which is an important step towards transforming cells into an invasive phenotype (5). These studies therefore, raise the question as to what effect RhoC inhibition would create *in vivo*. Our *in vivo* results showed that both lung inflammation and a large volume of lung metastases were present in animals which were implanted by peritoneal injection of either parental or shRNA-scrambled sequence (control) cell lines. In contrast, the lungs of mice implanted or injected with RhoC knockdown lines were free from any pathological findings, specifically lung metastases and inflammation in lung tissues and blood vessels (Fig.5). Furthermore, the level of angiogenesis in the localized tumors was assessed using CD31 antibody and these results showed a remarkable difference both in quality as well quantity of the microvessels in the tumors. The mice implanted with RhoC knockdown lines showed markedly fewer and less poorly developed microvessels as compared to the far greater in number and clearly defined vessels in parental or shRNA-control cell lines (Fig. 6).

The implications of the findings in this manuscript may provide a fertile area of research in head and neck squamous cell carcinoma. For instance, recent work has shown that matrix metalloproteinases, well known mediators of invasive tumor behavior, have been identified as a specific and critical player for the formation of lung metastasis (34, 35). Li *et al*, 2006, reported that the oncogene AF1Q which is responsible for primary breast tumor growth and pulmonary metastasis are at least, in part, regulated by MMP and RhoC expression (36). The remodeling of the actin cytoskeleton is a critical and important step in the formation of pulmonary metastasis due to changes in cell shape, polarity, cell interactions and eventual migration of the cancer cells. Interestingly, studies by Nelson *et. al*. have shown that the expression of MMP3 gene that induces

epithelial-mesenchymal transition in mammary epithelial cells is brought about by change in cell shape through Rac1 (also a member of the Rho family) mediated changes in cytoskeletal structure (37). Clearly, future studies elucidating the specific interactions between the MMP 2, 3 and 9 (major MMP proteins in head and neck squamous cell carcinoma) and RhoC are indicated and may prove very informative.

In conclusion, the findings presented in this manuscript illustrate that both *in vivo* and *in vitro* conditions RhoC plays an important role in head and neck cancer tumor progression and metastasis. With additional investigations and ongoing development of RhoC specific inhibitors, this may prove to be an important therapeutic target in this patient population.

Acknowledgments

The authors thank Dr. N. S. Mahfooz for critical reading of the manuscript, Dr. Thomas Carey for providing the cell lines for use in this work and the NCI SPORE program for grant support and the Department of Defense (BC083217 to J.C.B.).

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Figure Legends

Fig. 1 Lentivirus infected cell showing the GFP expression level. Panel (A) is presenting UM-SCC-11A: shRNA-scrambled sequence control (SR), RhoC knockdown clone 1 (c1) RhoC knockdown clone 2 (c2) and uninfected cells as the control (negative). Upper panel shows the histograms obtained by flow cytometry. Middle and lower panel representing the GFP labeled cells in fluorescent light and in bright light. Panel (B) is representation of UM-SCC-1. All other notations are the same as described above. As shown here a higher number of cells were infected as evident from GFP expression patterns.

Fig. 2 Quantitative RT-PCR of UM-SCC-1 and -11A shows the relative mRNA expression pattern of (A) RhoC, (B) Cdc42, (C) Rac1 and (D) Rac2 in parental, shRNA-scrambled control and RhoC knockdown clones 1 and 2 after infected with lentivirus using shRNA Strategies. Results were analyzed using $2^{-\Delta\Delta C_T}$ methods. A significant decrease in mRNA expression of RhoC knockdown clones were obtained while the expression of cdc42, rac1 and rac2 were remain unchanged. ($p < 0.05$).

Fig. 3 The extent of cell invasion through Matrigel. (A&E) Parental cell lines ;(B&F) shRNA-scrambled controls; (C&G) RhoC knockdown clones 1, (D&H) RhoC knockdown clones 2 of UM-SCC-11A and 1 respectively. Columns I and J, rates of invasion, bars, 95% CI $P < 0.05$.

Fig. 4 Effect of RhoC knockdown on cell motility. Panels A and B shows the slow movement of RhoC knockdown cells as compared to its parental or sh-RNA scrambled

control in UM-SCC-11A and 1 respectively. Bar graphs show the extent of percent motility ($p < 0.05$) reference point is the zero hour values.

Fig. 5 H & E stained slides shows the microscopic lung metastasis. Parental (A & D), shRNA-scrambled controls (B & E) RhoC knockdown clones (C & F). Number of cells obtained by culturing the lungs are depicted in G and H for UMS-CC-11A and 1 respectively ($p < 0.05$).

Fig. 6 A, B and C microvessel density assessment after staining with CD 31 antibody. Representative high-power fields from tumor developed in the parental (UM-SCC-1) (A), shRNA-scrambled sequence (B) and RhoC knockdown (C). Microvessels were smaller and poorly developed in RhoC knockdown clone.