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Functional genomics lead to new therapies in follicular lymphoma

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Abstract

Recent technological advances allow analysis of genomic changes in cancer in unprecedented detail. The next challenge is to prioritize the multitude of genetic aberrations found and identify therapeutic opportunities. We recently completed a study that illustrates the use of unbiased genetic screens and murine cancer models to find therapeutic targets among complex genomic data. We genetically dissected the common deletion of chromosome 6q and identified the ephrin receptor A7 (*EPHA7*) as a tumor suppressor in lymphoma. Notably, *EPHA7* encodes a soluble splice variant that acts as an extrinsic tumor suppressor. Accordingly, we developed an antibody-based strategy to specifically deliver *EPHA7* back to tumors that have lost this gene. Recent sequencing studies have implicated *EPHA7* in lung cancer and other tumors, suggesting a broader therapeutic potential for antibody-mediated delivery of this tumor suppressor for cancer therapy. Together, our comprehensive approach provides new insights into cancer biology and may directly lead to the development of new cancer therapies.

Keywords

genomic data; mouse model; therapies

Introduction

Cancer is a complex disease characterized by myriad genetic alterations that affect many pathways and processes.^{1–5} The cancer genome is altered at the nucleotide, chromosomal, and epigenetic levels. However, only a small number of mutations are required for malignant transformation; for example in acute myeloid leukemia only 2–3 mutations are sufficient to cause leukemia,⁶ implying that, while many of the changes identified may contribute to the malignant phenotype, they may be dispensable and therefore less likely to be important for cancer therapy. Identifying critical changes is complicated by significant inter- and intra-tumoral heterogeneity, and despite advanced statistical analyses, the descriptive data alone may not pinpoint the critical lesions that are amenable to therapy. Functional assays are needed to define the biological impact of genetic alterations and to

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Conflicts of interest

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understand their requirement for tumor development and also maintenance of the malignant cells. Therefore, this review emphasizes complementary approaches that include large-scale genomic data and genetic experiments to functionally annotate cancer genome data.

Cancer genomics and potential therapies

Genetic screens are unbiased experimental tools to simultaneously assess the biological activities of large numbers of genes. Well-designed screens are excellent assays to investigate complex genomic data, and the unbiased nature of this approach ensures that new lesions can be identified. Genetic screens are performed in different laboratories using different technologies. These include gain- and loss-of-function studies, both *in vitro* and *in vivo*, which may be performed on a one-by-one basis or as massively parallel experiments with pooled libraries.^{7,8,9} Our lab performs large-scale, pooled, short-hairpin RNA (shRNA) library screens, an ideally suited approach for prioritizing gene lists. For example, screening can be performed to identify functional candidates in large genomic regions affected by copy number alterations, or to study genes that have broad-acting biological function, such as those involved in epigenetic regulation, transcription factors, or microRNAs.^{1,9} The choice of screening experiment is a key consideration and the experimental system will determine the biological activity that can be identified. Clearly, an *in vitro* screen for transforming activities can only capture a subset of the biological processes involved in malignant transformation and will be blind to factors enabling immune escape – these would be considered false negative as they will not be found. Screens are further complicated by false positive “hits.” While repeated screens will eliminate most of them, some hits may have true activity in the screen system but lack significance *in vivo* or in the human disease. The latter may only be revealed upon individual validation in a genetically and pathologically accurate *in vivo* cancer model. In short, unbiased genetic screens are highly versatile tools to filter large-scale genomic data for subsequent *in vivo* validation studies.^{1,10,11}

Murine models of cancer enable direct functional studies of genetic alterations seen in human tumors (Fig. 1). In particular, mosaic models enable rapid assessment of genetic changes in a physiological context,^{11–13} an example of which is based on the adoptive transfer of virally transduced bone marrow cells into wild-type syngeneic recipient animals. This approach can be used to model several hematopoietic malignancies *in vivo*. Importantly, the ease of genetic modification avoids the time-consuming generation of mutant or transgenic animals for each allele to be studied. Our approach is based on the retroviral modification and transplantation of hematopoietic progenitor cells (HPCs) and allows rapid testing of several genes of interest.^{9,13,14} Moreover, using HPC derived from animals with different genetic backgrounds allows modeling of different forms of lymphoma, including the EμMyc murine model of Burkitt-like lymphoma,¹⁵ or the IμHABCL6¹⁶ and Eμ-CyclinD1,¹⁷ used to recapitulate the genetics of diffuse large B cell lymphoma (DLBCL) and mantle cell lymphoma, respectively. Most recently, we developed an adoptive transfer model based on vavBcl2 transgenic mice.¹² who develop a disease that resembles follicular lymphoma in genetics and pathological appearance, including evidence of somatic hypermutation and germinal center origin.¹² For the first time, this model enables the study of non-transformed follicular lymphoma (FL) to examine genetic and cellular

interactions involved in lymphomagenesis, treatment response, and malignant progression. In conjunction with human genomic data and unbiased genetic screens, these murine models can provide direct evidence of pathogenic drivers and genetic requirements in human cancer.

We applied a functional genomics strategy to identify driver mutations in FL,¹ the most common indolent form of non-Hodgkin lymphoma. The cytogenetic hallmark of FLs is the chromosomal translocation t(14:18)(q32;q21) that results in constitutive expression of the anti-apoptotic gene *Bcl2*.¹⁸ However, additional genetic lesions are required to induce lymphomagenesis or disease progression, which occurs in 50% of cases.^{19,20} For example, c-MYC amplification, loss of p53, and deletions of chromosome 6 have been linked with disease progression and reduced survival.^{21–24} Overall, the median survival of patients with FL is approximately 8–10 years and is limited in 50% of cases by the transformation into aggressive B cell lymphoma; the remainder of patients show a pattern of incessant disease relapse following chemotherapy that eventually outruns the patients marrow reserve, leading to transfusion dependence and recurrent infections. The inclusion of the anti-CD20 antibody (Rituxan®) in the treatment of FL fifteen years ago has been the last major improvement and has significantly impacted patient outcomes.^{25,26} Despite this advance, bone marrow transplantation remains the only curative option in suitable patients. It has been argued that survival times in this cancer are long, and that, with an elderly patient population, further improvements may be hard to accomplish. However, we think that new insight into the pathogenesis of FL can lead to more effective and potentially less toxic alternatives to the current combinations of chemotherapy and Rituxan.

Scientifically, FL has not received much attention compared to aggressive lymphomas. In part, this is due to a lack of non-transformed FL cell lines and adequate animal models. Accordingly, most studies have sought to correlate patient features and individual genetic changes with the outcome under available therapy, and to inform doctors' decisions on whether and when to initiate treatment.^{27,28} Only recent advances in sequencing technology have provided new insights into the genetic make-up of FL, including the unexpected finding that the most frequently mutated genes in FL are involved in epigenetic control of gene expression (e.g., *EZH2*, *MLL2*, *MEF2B*, and *CREBBP*).^{29,30} Among other findings, recurrent mutations in *B2M* and *CD58* implicate immune escape, and activation of B cell receptor signals that are most likely endogenous or mutational also contribute to FL pathogenesis.^{31,32} In addition to these recurrent somatic mutations, genomic analyses reveal recurrent chromosomal gains and losses, including deletions of chromosome 6q, which occur in approximately 25% of FLs and are associated with poor prognosis.^{1,33} Typically, 6q deletions are large and hemizygous, and thus the genetic target(s) of chromosome 6q loss have long been an enigma. Recently, several tumor suppressor genes were identified for diffuse large B cell lymphoma that are encoded on chromosome 6q, including *TNFAIP3*^{34,35} and *PRDM1/BLIMP*.³⁶ The pattern and size of 6q deletions indicates the existence of multiple tumor suppressor activities in this region.

To search for genes that may contribute to FL development, we designed a retroviral shRNA library against all genes encoded within regions of loss on chromosome 6q, and each gene was targeted by two to five different short hairpins. An unbiased genetic screen was performed for cooperation with *BCL2* in B lymphocytes *in vitro* to identify shRNAs that

provide proliferative advantage. The screening identifies several interesting candidates that were validated individually. *TNFAIP3* was immediately confirmed as a tumor suppressor in lymphoma and *EPHA7* was identified as a new candidate gene. A short splicing variant of *EPHA7* that encodes a soluble protein is normally expressed in B lymphocytes and is lost in up to 75% of FLs.³⁷ We used vavPBcl2 chimeric mouse model of FL to test and validate *EPHA7* activity in a specific tumor context, and readily confirmed that a loss of *EPHA7* promotes FL development, and conversely, its re-expression slows tumor growth *in vivo*.

Ephrin receptors are a large family of receptor tyrosine kinases involved in cell–cell signaling, and alterations in these genes have been implicated in solid cancers.^{38,39} Specifically, ephrin receptors are activated by binding ephrins (ligands) that are expressed on the cell surface. These ligand–receptor interactions stimulate formation of duplex and higher order ephrin–receptor clusters, which are involved in cell signals. Notably, a cellular signal emanates in both directions from ligand and receptor, and changes cell behavior. This signaling pathway has been implicated in mechanisms controlling brain size, axon guidance, and retina formation.^{40,41} Within the family of ephrin receptors, *EPHA7* is notable for the existence of a short splice variant that encodes a truncated protein (EPHA7TR). The truncated EPHA7TR can be shed from the cell surface and acts as a dominant inhibitor of the full-length receptor that forms inactive heterodimers with full-length ephrin receptors. This surprising mode of action for *EPHA7* has previously been implicated in closure of the neural tube during embryonal development.⁴² While *EPHA7* is highly expressed in lymphoid tissues, its physiological role in B-cell development or activation has not been explored. Importantly, the cell-extrinsic mode of action suggests that exogenous administration may be able to restore *EPHA7* function.

In follicular lymphoma, *EPHA7* is deleted and silenced by promoter methylation in over 70% of the tumors; this percentage may be even higher in aggressive lymphomas. *EPHA7* acts in the pathogenesis of FL by binding and blocking the activity of the EPHA2 receptor, which then inactivates ERK and SRC kinase pathways. Notably, treatment of cultured or xenografted lymphoma cells with *EPHA7* inhibits ERK and SRC activity and causes cell death, suggesting a potential therapeutic application for *EPHA7* in lymphoma therapy. Moreover, in analogy with the concept of oncogene dependence,⁴³ it has been proposed that tumor cells are exquisitely sensitive to the restoration of tumor suppressor genes. Indeed, studies in genetically engineered mice where a tumor suppressor can be re-activated indicate powerful anti-tumor activity.^{44,45} For example, restoring p53 function has been demonstrated to have a powerful and tissue-specific mechanism that suppresses tumorigenesis,⁴⁴ supporting the idea that pharmacological reactivation of tumor suppressor genes is a therapeutic possibility. However, most tumor suppressors act cell intrinsically, and are mutationally lost and not amenable to exogenous restoration. Exogenous administration of *EPHA7* may represent a rare instance where tumor suppressive activity can be restored.

We tested different routes for administering the soluble EPHA7 protein for lymphoma therapy. While local administration was extremely powerful, this is not a satisfying therapy for a systemic disease like lymphoma. Systemic intravenous administration showed antitumor activity and was well tolerated, however it proved far less effective than the local

application. Finally, we explored the possibility of using the anti-CD20 (Rituximab) antibody to deliver effective concentrations of *EPHA7* to the lymphomas *in vivo*. This approach was highly effective and combined the intrinsic anti-tumor effect of the anti-CD20 antibody with the specific tumor suppressor effect of *EPHA7* that is able to shut down ERK and SRC signals in lymphoma cells. The fusion of *EPHA7* with the anti-CD20 antibody represents an improvement over anti-CD20 alone, and reflects the specific sensitivity of cancer cells towards the restoration of a tumor suppressor.

Recently, *EPHA7* has also been implicated in other cancers: its expression is lost in DLBCL, acute B lymphoblastic leukemia (B-ALL), sarcoma, colon, and prostate cancers.^{46–49} Recently, deep sequence analyses revealed common mutations in *EPHA7* in small cell lung cancer, melanoma, head and neck carcinoma, and other cancers^{50–55} (Fig. 2 and Table 1). Together, these data indicate an important role for ephrin signaling and *EPHA7* in a spectrum of human cancers. We are currently testing the potential of *EPHA7* administration to treat other tumor types.

Perspective

Cancer genomic studies improved cancer therapy. In the past few years, drugs targeting specific genetic alterations have been successfully developed.^{56,57–59} The success of these therapies is based, at least in part, on the continuous requirement of the initiating oncogenic signals for tumor maintenance. Recent sequencing studies have highlighted the important role of recurrent somatic mutations in individual genes. Somatic mutagenesis is not the only process that shapes the cancer genome. Additional changes such as gains and losses of chromosomal material or activation and silencing of complex transcriptional programs are likely just as important. However, the functional analysis of such large-scale alterations remains a major challenge. We described a highly efficient strategy to prioritize large gene sets altered in human tumors that is based on combination of unbiased genetic screens and the use of highly versatile animal models of cancers. In this manner, we can identify key driver genes within large regions of genomic loss that lend themselves to new therapies.

Chromosomal gains and losses are not precise mutagenic events like point mutations. However, the recurrent nature of these lesions indicates that they confer fitness advantage and contribute to tumor development or progression. Chromosomal aberrations likely affect multiple tumor suppressor activities that may produce cooperative effects,⁶⁰ which is the case with the large 6q deletions seen in lymphoma. Notably, the restoration of one tumor suppressor (*EPHA7*) is sufficient to produce dramatic therapeutic effects. The imprecise nature of these lesions may also affect genes whose inactivation is not beneficial to the tumor cell,^{61,62} potentially resulting in collateral sensitivity of tumor cells that have lost one gene copy towards pharmacological inhibitors.

Does the *EPHA7* tumor suppressor have therapeutic activity beyond lymphoma? Recently, multiple genomic studies have indicated that *EPHA7* is a mutational target across multiple cancers (Fig. 2), opening the possibility to extend the therapeutic use of *EPHA7* to different tumors. Notably, *EPHA7* is one of very few soluble tumor suppressors that lend themselves to exogenous administration. We have been able to specifically deliver *EPHA7* to

lymphomas by fusing *EPHA7* to the anti-CD20 antibody already used in lymphoma therapy. Similarly, antibodies have been used to deliver toxins or radioactive compounds to tumors cells. The targeted delivery of a tumor suppressor has an important conceptual advantage over the targeted delivery of broad acting toxins. Namely, cancer cells are especially sensitive to the restoration of a tumor suppressor that has been lost during development of the tumor, a phenomenon that has been called “tumor suppressor hypersensitivity,”⁴⁵ which adds to the specific anti-tumor effect of the fusion construct.

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Figure 1.

A combination of powerful tools to functionally annotate genomic data. Analysis of cancer genomic data provides a list of relevant alterations for functional validation. Genetic screens are versatile tools to simultaneously test many genes for subsequent validation in a physiological context.

Figure 2. *EPHA7* is mutationally altered in several human cancers. Recent papers report that *EPHA7* is mutated or deleted in different cancers including melanoma, small cell lung cancer, and lymphoma, implying a potential application of *EPHA7*-based therapies for these cancers.

Table 1

Summary of EPHA7 reported genetic alterations

	Cancer Type	Alterations	Frequency	Reference
1	Skin cutaneous melanoma	Mutations	13.2	Hodis <i>et al.</i> ⁵²
2	Lung adenocarcinoma	Mutations	9.3	Imielinski <i>et al.</i> ⁵³
		Deletions	1.1	
		Amplifications	0.5	
3	Small cell lung	Mutations	10.3	Peifer <i>et al.</i> ⁵⁰
4	Follicular lymphoma	Deletions	11	Oricchio <i>et al.</i> ¹
5	Cell Line Encyclopedia	Mutations	6.5	Barretina <i>et al.</i> ⁴⁹
		Deletions	2.2	
6	Prostate adenocarcinoma	Deletions	6.6	Grasso <i>et al.</i> ⁵⁴
7	Head and neck	Mutations	5.4	Stransky <i>et al.</i> ⁵¹
8	Small cell lung	Mutations	4.8	Rudin <i>et al.</i> ⁵⁵
9	Lung adenocarcinoma	Mutations	3.7	Ding <i>et al.</i> ⁶³
10	Colorectal	Mutations	2.8	Seshagiri <i>et al.</i> ⁶⁴
11	Medulloblastoma	Mutations	2.7	Robinson <i>et al.</i> ⁶⁵
12	Breast	Mutations	2	Stephens <i>et al.</i> ⁶⁶
13	Kidney	Mutations	2	Guo <i>et al.</i> ⁶⁷
14	Breast	Mutations	1.9	Banerji <i>et al.</i> ⁶⁸
15	Prostate adenocarcinoma	Mutations	0.9	Barbieri <i>et al.</i> ⁶⁹
16	Medulloblastoma	Mutations	0.8	Jones <i>et al.</i> ⁷⁰

Source: www.cbioportal.org