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Genomics screens for metastasis genes

Jinchun Yan · Qihong Huang

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Abstract Metastasis is responsible for most cancer mortality. The process of metastasis is complex, requiring the coordinated expression and fine regulation of many genes in multiple pathways in both the tumor and host tissues. Identification and characterization of the genetic programs that regulate metastasis is critical to understanding the metastatic process and discovering molecular targets for the prevention and treatment of metastasis. Genomic approaches and functional genomic analyses can systemically discover metastasis genes. In this review, we summarize the genetic tools and methods that have been used to identify and characterize the genes that play critical roles in metastasis.

Keywords Metastasis · Genomic screen · Functional genomics · Next-generation sequencing

1 Introduction

Metastasis is the formation of tumors at distant sites following the spread of cancer from a primary site [1, 2]. When cancer is detected before it has spread, it can often be treated successfully with surgery or local and systemic adjuvant chemoradiation therapy. However, when it is detected after it has metastasized, treatments are much less successful [3]. Furthermore, due to the lack of diagnostic tools, many patients in whom there is no evidence of metastasis at the time of initial diagnosis develop metastases later [4, 5]. Metastatic lesions, rather than the primary tumor, are responsible for most cancer deaths and have consequently become the most feared aspect of cancer [4, 5]. Hematogenous metastasis occurs via multiple steps starting with cancer cells escaping from the primary tumor and entering the blood stream (intravasation). Cells that are able to survive in the circulation come to rest in capillaries at a new site, then exit from capillaries into surrounding tissues (extravasation), initiating the formation of micro-metastases. The development of new blood vessels (angiogenesis) then enables the formation of secondary tumors [6–10]. The metastatic process is also very inefficient and only an extremely small percentage of tumor cells are able to complete all the steps in this complex process and survive at secondary organs [11–13]. Completion of this journey requires fine coordination among the genes involved at each step of the process [14–17]. The dissemination of cancer cells and growth of metastases depend on the balance between genetic programs in tumor cells and host's genetic background that promote or suppressor metastasis [18, 19]. Identification and understanding genome-wide alterations associated with this pathological process are vital to elucidating the complexity of the metastatic process. The recent development of genomics approaches allows the identification of metastasis genes at the genome-wide level. In this review, these genomics screens for metastasis genes are discussed (Table 1).

J. Yan (✉)
University of Washington Medical Center,
1959 N. E. Pacific Street,
Seattle, WA 98195, USA
e-mail: jyan@u.washington.edu

Q. Huang (✉)
The Wistar Institute,
3601 Spruce Street,
Philadelphia, PA 19104, USA
e-mail: qhuang@wistar.org

2 Cytogenetics in metastasis

Efforts to identify genes involved in the metastatic process at the genome-wide level started with cytogenetics

Table 1 Genomics screens for metastasis genes

Genomic and genetic approaches	Application in metastasis
Cytogenetic	Karyotypic changes and LOH in metastatic cells and tissues
Microcell-mediated chromosome transfer	Metastasis suppressors
Gene expression profiling	Metastasis genes deferentially expressed in tumor cells and tissues
Differential colony hybridization	
Microarray and Gene chip	
Quantitative trait loci analysis	Polymorphisms in genetic background contributing to metastasis
Functional genomics	Functional identification of metastasis genes at genome-wide level
Genome-wide RNAi, cDNA, and microRNA screens	
Next-generation sequencing	High-throughput Identification of somatic mutations, genetic variations, epigenetic regulations in metastasis cell and tissue
Whole-genome sequencing	
Whole-genome CGH	
Whole-genome epigenetics	

technologies before the sequencing of the human genome was complete. The karyotypes of tumor cell lines or tumor samples that exhibited metastatic or non-metastatic phenotypes were compared [20–26]. These comparisons were made both between the paired cell lines with differential metastatic potential, as well as between the metastatic subclones and non-metastatic parental cell lines [20–22]. Clinical samples of primary tumors and their matched metastatic samples were also compared [23–26]. These studies identified chromosome aberrations including gains, losses, translocations and loss of heterozygosity, all of which may potentially be associated with metastasis phenotypes. For example, loss of heterozygosity for chromosome 11 and 13 has been found to be associated with metastasis and poor survival in breast cancer [27]. Although these studies did not identify specific gene(s) that either promote or suppress metastasis or elucidate the functions and mechanisms of metastasis genes and pathways, they provided genetic bases for complex metastatic phenotypes.

3 Microcell-mediated chromosome transfer

The studies that identified karyotypic changes and loss of heterozygosity as important in metastasis sparked efforts to isolate specific genes that regulate metastatic process. The microcell-mediated chromosome transfer (MMCT) method, which was developed to introduce chromosomes into intact recipient cells to identify tumor suppressors and senescence genes, was used to introduce chromosomes containing metastasis suppressor gene(s) into tumor cells [28]. Human/mouse hybrid A9 donor cells carrying a single human chromosome with a selection marker were fused with recipient cells to generate microcell hybrids. These microcells were characterized using cytogenetic methods and/or PCR to define the addition or deletion of donor and recipient cell chromosomal regions. Spontaneous metastatic ability of these microcell hybrids was assayed *in vivo* [29]. This approach successfully isolated metastasis suppressor genes on chromosomes 1, 6, 7, 8, 10, 11, 12, 16, and 17 [30–43]. The following are some of the examples of genes that were identified by this method:

3.1 BRMS1

MMCT was used to transfer chromosome 11 into the highly metastatic breast cancer cell line MDA-MB-435 [16]. Differential display comparing the parental cells with the chromosome 11 containing cells led to the isolation of breast cancer metastasis suppressor 1 (BRMS1). Subsequent studies showed that BRMS1 can also suppress metastasis in models of non-small cell carcinoma, ovarian, melanoma and bladder cancer [44–47]. Immunohistochemical staining further indicated that BRMS1 expression is correlated with survival in breast cancer [48, 49] and non-small cell lung carcinoma [46].

3.2 KAI1

KAI1 is a member of tetraspanin family that was identified by MMCT of transfer of human chromosome 11 into rat prostate cancer cell lines [50]. It has been shown that KAI suppresses metastasis in prostate and breast cancer models [50–52]. The expression of KAI1 is downregulated in the clinical samples of prostate, breast, ovarian, colorectal, and non-small cell lung cancer [53–56].

3.3 MAP2K4

MAP2K4, a member of MAP kinase family, is located on chromosome 17. It was identified as a metastasis suppressor in prostate cancer by MMCT and positional cloning [57]. Its expression is downregulated in breast, pancreatic, and gastric cancer and correlates with poor prognosis [58–60].

Overexpression of MAP2K4 also reduced onset and incidence in ovarian and prostate cancer models [61]

3.4 KISS1

KISS1 was also identified by MMCT in human metastatic melanoma cells [17, 62]. KISS1 and its G-protein-coupled receptor GPR54 have been shown to regulate puberty [63], demonstrating that KISS1 has physiological functions besides its roles in cancer development. KISS1 suppresses metastasis in melanoma, breast, pancreatic, and ovarian cancer models [64–66]. High expression of KISS1 is correlated with survival in various tumor types [59, 67–74].

4 Differential gene expression in metastasis

It has been hypothesized that genetic programs in cancer cells control the metastasis process and that the genes that regulate this process are expressed differentially in cancer cells with varying metastasis potentials. Differential colony hybridization and microarray methods were used to identify genes that are differentially expressed.

4.1 Differential colony hybridization

This method allows comparison of gene expression from two different cell populations such as two cells derived from the same parental cell line that have different metastatic potential. The first metastasis suppressor, NM23, was found using differential colony hybridization in matched murine high and low metastatic cell lines that were derived from the parental murine melanoma cell line K-1735 [75]. It has since been shown that two family members of NM23, NM23-H1 and NM23-H2, suppress metastasis in multiple tumor types [76] and that expression of these genes is inversely correlated with poor survival in multiple cancer types including breast, ovarian, melanoma, gastric and non-small cell carcinoma [77–79]. More importantly, small molecules such as dexamethasone and medroxyprogesterone have been shown to induce NM23 expression *in vitro* and suppress metastasis in animal models [80, 81], demonstrating that genetic programs can be manipulated in cells to inhibit the metastatic process.

4.2 Microarray

In combination with microarray analysis which detects gene expression at the genome-wide level, a strategy similar to that used for the identification of NM23 has been used to identify genes associated with metastasis to various secondary organs. Cell lines with a preference for bone metastasis were isolated from the parental human breast cell line MDA-MB-231 and compared, using microarray analysis, to MDA-MB-231 cells with low potential for bone metastasis [82]. A gene expression signature associated with osteolytic bone metastasis was identified from the microarray analysis. Among the genes in the signature, IL11, CTGF, CXCR4, and MMP1 were most highly differentially expressed and were shown to cooperate functionally to promote breast cancer metastasis to bone [82]. Similarly, microarray analysis was also performed in cells with high and low metastatic potential to lung [83]. A lung metastasis gene expression signature was also identified. Id1, MMP1, CXCL1, PTGS2, VCAM1, and EREG were among the genes that promote lung metastasis in animal models and were the most significant genes that differentiate breast cancer patients with lung metastasis from other breast cancer patients. Id1 is especially important because it can promote lung metastasis by itself in animal models and is highly expressed in samples from breast cancer patients with lung metastasis [83].

Similar to the isolation of genes specific to lung metastasis, human breast cancer cells that preferentially metastasize to brain were isolated and analyzed using gene expression profiling [84]. Among the genes that were either upregulated or downregulated, the expression of 17 genes was correlated with brain cancer relapse. Among these 17 genes, ST6GALNAC5 was found to specifically mediate brain metastasis in breast cancer by promoting adhesion of breast cancer cells to brain endothelial cells and mediating passage through the blood–brain-barrier. COX2 and the epidermal growth factor receptor ligand HBEGF also promote breast cancer cell extravasation and both brain and lung metastasis.

Mouse tumor cells have also been used to identify metastasis-promoting genes. Gene expression profiling was performed on 67NR, 168FARN, 4TO7, and 4T1, four cell lines that were isolated from a single murine mammary tumor and have differential metastatic potentials [85, 86]. Twist, a master regulator of embryonic morphogenesis, was found to be upregulated in highly metastatic 4T1 cells but downregulated in non-metastatic cells. Twist was shown to promote breast cancer metastasis by promoting epithelial-mesenchymal transition (EMT) [86].

Gene expression profiling has thus proved to be a powerful tool to isolate genes regulating metastasis in various types of tumor [87–95]. It is anticipated that careful selection of cell lines and clinical samples for profiling will yield additional genes and noncoding molecules that play critical roles in the metastatic process.

5 Quantitative trait loci analysis for metastasis polymorphisms

It has been shown that an individual's genetic background can have a significant impact on cancer progression [96,

97]. However because the contribution of each host gene to the metastatic phenotype is small, it is difficult to identify candidate genes and polymorphisms. To address this problem, Hunter and colleagues developed an approach to determine the influences of tumor behavior by mouse background. The polyoma middle-T mouse (PyMT), which expresses the mouse polyoma virus middle-T antigen in the mammary epithelium was used to determine the contribution of genetic background to metastasis [98]. When PyMT mice were bred to FVB/N mice, mammary tumors metastasized to lung with high frequency. In contrast, significant variation in tumor metastasis appeared in the F1 progeny when PyMT mice were bred to other strains including DBA2/J and NZB/B1NJ. Genetic mapping identified Rap1-Gap molecule Sipa1 as a candidate for the metastatic phenotype [98]. Even a minor change in Sipa1 expression affects the capability of tumor cells to form lung metastasis [98]. This study confirmed that an inherited polymorphism can play an important role in cancer progression to metastasis.

The completed sequencing of human and mouse genomes allows genome-wide association studies (GWAS) to identify potential polymorphisms that contribute to disease susceptibility including cancer. Susceptibility genes have been identified in most cancer types, however little is known regarding susceptibility to metastasis, which has been shown to be a complex trait that involves the contribution of multiple genes. Common genetic variants in different individuals were examined to determine whether any variant is associated with metastasis. RRP1B, Brd4, and SIPA1 have been identified as metastasis susceptibility genes in human breast cancer [98–100]. Advances in quantitative trait locus analysis and GWAS are likely to enable identification of additional metastasis susceptibility genes with additive or nonadditive interactions in various types of cancer.

6 Genome-wide RNAi and cDNA screens for metastasis genes

Much of our understanding of metastasis has been obtained through reverse genetic approaches in which a gene of interest is investigated for its roles in the metastatic process. This approach has yielded important insights into the mechanisms of metastasis. However, this knowledge is restricted to a small number of genes. Forward genetic screening offers a possible solution to this challenge, enabling the identification of metastasis genes without *a priori* knowledge of their functions. The development of RNA interference (RNAi) technology allows the performance of forward genetic screens in mammalian cells. RNAi, cDNA, and microRNA expression libraries have been used in cell culture and animal models to identify genes that promote and suppress metastasis.

6.1 RNAi library

A genome-wide lentiviral RNAi library consisting of short hairpin RNAs (shRNAs) targeting 40,000 mouse genes was introduced into non-metastatic mouse mammary tumor 168FARN cells [101]. These cells were then transplanted into mouse mammary fat pads where they would normally remain unless RNAi knock-down of a metastasis suppressor, which can complement the metastatic deficiency of 168FARN cells, enabled the cells to disseminate from the mammary fat pad and form metastases in the lung. In essence, the mice are used as “cell sorters” and the development of lung metastases serves as the selection system. The incorporation of shRNAs into the host genome after lentiviral infection makes it possible to easily retrieve shRNAs from the positively selected cells by PCR. Tumor cells metastasized to the lung were visualized by bioluminescence using the Xenogen system, isolated, and the RNAi was retrieved to enable identification of Krüppel-like factor 17 (KLF17) as the target gene. The suppression of transcription factor KLF17 promotes tumor cell invasion and leads to EMT in mammary epithelial cells and that it suppresses the expression of the metastasis regulator Id1 by directly binding to its promoter region. Id1 has been identified as an important molecule in lung metastasis in breast cancer [83], and our studies demonstrated that KLF17 regulates metastasis largely through Id1 suppression [101]. Since KLF17 expression is significantly downregulated in breast cancer metastasis, the combination of KLF17 and Id1 expression may serve as biomarkers for breast cancer metastasis [101].

A genome-wide RNAi library was also used in 3D cell culture to identify the metastasis suppressor GAS1 in melanoma [102]. A mouse short hairpin RNA library was introduced into poorly metastatic B16-F0 mouse melanoma cells, which were subsequently grown in collagen and matrigel. Colonies were selected and shRNAs were retrieved. Twenty-two genes were identified from the screen whose knockdown promotes metastasis without affecting primary tumor growth. GAS1 was further characterized and shown to suppress metastasis by promoting apoptosis in the tumor cells disseminated to secondary organs. GAS1 is downregulated in metastatic human cell lines and clinical samples.

6.2 cDNA library

Similarly, a cDNA library in a lentiviral vector was introduced into non-metastatic 168FARN cells, which were then injected into mouse mammary fat pads [103]. Development of lung metastasis was used as a selection system. Metastatic cells in the lung were isolated and cDNAs were retrieved by PCR. Disulfide isomerase ERp5 was identified as a metastasis-promoting gene [103]. This study demonstrated

that ERp5 promotes metastasis through the activation of ErbB2 and phosphoinositide-3 kinase pathways [103]. Activation of these pathways subsequently stimulates RhoA and β-catenin which mediate the migration and invasion of tumor cells [103].

6.3 MicroRNA expression library

A microRNA expression library consisting of approximately 450 microRNAs was also used to identify metastasis-promoting microRNAs [104]. This library was introduced into non-invasive human breast cancer MCF7 cells, which were subject to transwell invasion assays. A family of microRNAs—miR-373, 520c and 519e—which share similar seed sequence, was found to stimulate migration and invasion *in vitro* and metastasis in a mouse xenograft model [104]. MicroRNA miR-373 is also upregulated in breast cancer metastasis samples [104].

Although these screens were performed in melanoma and breast cancer model, the same approaches can also be used to identify metastasis genes in other types of cancer. The genes identified using these approaches can compensate for the metastasis deficiency of the cell lines used in the screen [105]. Thus the characterization of the cell lines, especially deficiencies in steps of metastasis process in the cell lines used for the screen, is important. Whether there are “master” regulators of metastasis, which regulate every step in the metastatic process, has yet to be determined. Functional genomics screening is also an important approach to separate metastasis drivers from passengers among the metastasis candidates that emerge from the studies using genome-wide methods such as whole-genome sequencing and microarray.

7 Next generation sequencing for metastasis genes

The development of massively parallel sequencing technology enables the high-throughput detection of somatic mutations and genetic variations in primary tumors and metastatic samples. This method is used increasingly in various types of cancer to identify genetic variations that are specific to metastasis and serve as drivers of metastasis.

7.1 Melanoma

A comparison of the genomes of a primary acral melanoma, matching regional lymph node metastases and normal samples prior to treatment revealed similarities in the mutation rate and spectrum, somatic mutations in the coding region, single nucleotide variation, copy number alteration and loss of heterozygosity, and structural variation such as translocation, insertion and deletion between primary and metastasis samples [106]. Two *de novo* mutations were detected only in the metastatic sample but not in the primary tumor [106]. One such mutation was found in the coding region of WNT1, which is a member of the Wnt-β-catenin pathway known to drive metastasis in melanoma. The other mutation disrupts the splicing site of SUPT5H which is a regulator of transcription elongation. Although lymph node metastasis is mechanistically different from metastasis to distant organs, this study raises at least two possibilities: (1) lymph node metastases represent subclones of primary tumors, in which case the mutations specific in metastasis are potential metastatic drivers or (2) the genetic lesions were acquired by the tumor cells early in tumorigenesis, in which case the mutations in those metastatic samples have no function in metastasis. Sequencing of additional pairs of primary and metastatic samples and functional studies of the mutant genes are required to reach conclusions.

Whole genome array-comparative genomic hybridization (CGH) profiles of 25 primary cutaneous and 61 metastatic melanoma specimens were analyzed using integrative genome comparison to identify 30 potential metastasis-promoting genes located in 32 genomic regions that are altered in metastatic samples [107]. Functional assays further confirm the pro-invasive properties of *MET*, *ASPM*, *AKAP9*, *IMP3*, *PRKCA*, *RPA3*, and *SCAP2* genes among these candidates. This study demonstrated that the combination of integrative genomic analysis and functional characterization is a powerful approach to discover metastasis regulatory genes.

7.2 Colorectal cancer

The exons of 1264 genes associated with cancer pathways in 21 pairs of primary colorectal carcinoma and their matched hepatic metastases were sequenced [108]. There are substantial differences in the coding mutation profiles between paired primary and metastatic samples indicating that there is heterogeneity among primary tumors and their metastases. Whether these *de novo* mutations have any role in metastasis development remains to be investigated.

Single-nucleotide polymorphism (SNP) analyses in the primary tumor samples of colorectal cancer patients with or without liver metastasis were performed [109–111]. Compared with the non-metastatic samples, primary tumors with known liver metastasis showed gain of chromosome 7p, 8q, 13q, and 20q and loss of chromosome 1p, 8p, 9p, 14q, and 17p. These studies also provide candidate genes such as SMAD4, DCC, TP53, TPD52, FABP5, MAP2K4, LLGL1, and FBLN1 that potentially regulate the metastatic process.

7.3 Prostate cancer

7.3.1 Genome sequencing and genome-wide CGH for metastasis genes in prostate cancer

It has been shown that primary prostate cancer is genotypically heterogeneous [112]. Whether metastatic prostate cancer is from a single clone or multiple clones of primary tumor is not known. Genome-wide CGH was performed on 85 anatomically separate cancer sites from 29 patients with metastatic prostate cancer [113]. The results showed that most metastases arise from a single clone of primary tumor and have a stable copy number change. This study raises the hope that it is possible to eradicate or halt the development of metastatic tumor in prostate cancer.

In order to identify the genes involved in metastasis in prostate cancer, high resolution of CGH and next generation sequencing of mutational analysis of cancer-related genes were performed on metastatic prostate tumors in three studies [114–116]. Amplification of regions of chromosome 5, 7, 14, and X and deletions of regions of chromosome 8, 10, 13, and 21 were identified. Copy number changes that were identified in metastatic samples in these studies include SKP2, PTEN, and P53 and mutations include P53 and BRCA2 [116]. These genes are metastasis candidate genes in prostate cancer that required further characterization.

Genome-wide exome sequencing on 23 metastatic prostate tumors also revealed additional recurrent mutations in many genes, including SDF4, PDZRN3, DLK2, FSIP2, NRCAM, MGAT4B, PCDH11X, GLI1, and KDM4B, which leads to the generation of hypotheses for further studies in prostate cancer metastasis [117].

7.4 Pancreatic cancer

Whole-genome sequencing was performed on the primary pancreatic tumors and metastatic pancreatic tumors from lung, live and peritoneum [118]. Most chromosome rearrangements are shared between primary and metastatic tumors indicating that most genome structure variation occurred in primary tumors before metastasis was evident [118]. Similar to prostate cancer, most pancreatic metastases appear to be clonal [118]. There was also ongoing evolution of gene rearrangements during metastasis in one patient indicating that genomic instability occurs throughout the life cycle of pancreatic cancer, and that two or more subclones from primary tumor are able to seed the metastatic tumors.

Mutational analysis in matched primary and metastatic pancreatic tumors from two or more organ sites illuminates the clonal evolution of metastatic pancreatic cancer [119]. These analyses indicate that metastatic clones evolve from those within primary tumors and genetic heterogeneity of metastatic tumors reflects that of primary tumors. Analysis of the timing of the evolution suggests that metastasis is a late event in pancreatic cancer development. At least a decade is required for the tumor cells to occur after they acquire the initiation mutations. It takes at least 5 years for these non-metastatic tumor cells to disseminate and patients die an average 2 years thereafter. These results indicate the importance of early detection and treatment in the prevention of death from metastatic pancreatic cancer.

7.5 Breast cancer

Whole-genome sequencing was used to detect mutations that potentially regulate metastasis in breast cancer. Genomic analysis of a basal-like primary breast cancer, its matched brain metastases and normal tissues, and a xenograft derived from the primary tumor revealed that the mutations of NRK, PTPRJ, WWTR1, and CHGB are highly enriched in breast cancer metastases [120]. Two *de novo* mutations specific in metastases, SNED1 and FLNC, were also identified from the analysis; 96.11 % of copy number alterations from the primary tumor were also found in metastases indicating that most copy number alterations are conserved during disease progression to metastasis. The genome analysis on this patient suggested that metastasis derived from a small subset of cells in the primary tumor. Larger numbers of samples are required to validate the conclusions of these results.

A similar study of matched primary and metastatic samples from an estrogen receptor-positive breast cancer patient identified 19 somatic coding mutations that are specific for metastasis suggesting that these mutations are potential drivers for metastasis development, or result from radiation therapy the patient received before the collection of the metastatic tumor [121]. Further functional studies and analysis in larger number of patients will identify metastatic drivers in breast cancer.

8 Future directions

Because of the development of new technologies, novel metastasis candidate genes will be identified in both clinical samples and laboratory models at a rapid speed. Functional studies are required to differentiate metastatic drivers from passengers among all these candidates, and functional genomics analysis will become increasingly important in metastasis research. Stromal cells in tumor tissues are also critical in metastasis process, and the development of new technologies will make it easier to identify genes in the tumor microenvironment that regulate metastasis. Metastasis genes identified through these approaches can potentially serve as prognostic or diagnostic biomarkers and therapeutic targets.

The advances of next generation sequencing make it conceivable that the patients' tumor samples and normal controls will be sequenced in clinics, thus providing timely information on genome structure and gene expression. Genetic structure variations, somatic mutations, and alterations in epigenetic regulation in the tumor and genetic background of patients will be detectable. The integrative analysis of all these data will provide both prognostic and diagnostic value and will guide both treatment and patient follow-up. Personalized medicine will also make it possible to treat patients based on the comprehensive analysis of both the tumor and the patient's genetic background. Patients without metastasis when they are diagnosed will be treated to prevent metastasis, whereas patients with metastasis will be treated according to the genetic types of metastases. Metastasis will eventually become a curable or manageable disease.

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