Summary. Metastasis is a major cause of cancer mortality. Metastasis is a complex process that requires the regulation of both metastasis-promoting and metastasis suppressor genes. The discovery of metastasis suppressor genes contributes significantly to our understanding of metastasis mechanisms and provides prognostic markers and therapeutic targets in clinical cancer management. In this review, we summarize the methods that have been used to identify metastasis suppressors and the potential clinical impact of these genes.

Key words: Metastasis, Metastasis suppressor, RNA interference, Functional genomics, Next generation sequencing

Introduction

Metastasis is a process in which primary tumors disseminate to secondary organs. It is a complex, inefficient, but deadly process (Paget, 1889; Weiss, 1990; Welch et al., 2000; Chambers et al., 2000; Fidler, 2003; Parker and Sukumar, 2003; Gupta and Massague, 2006; Steeg, 2006; Hurst and Welch 2011; Valastyan and Weinberg, 2011). The completion of this process requires coordination of the activation of metastasis-promoting genetic programs and the inhibition of metastasis-suppressing programs in tumors, as well as tumor microenvironments that allows cancer cells to escape primary sites and grow in secondary organs (Leone et al., 1991; Lee et al., 1996; Seraj et al., 2000; Muller et al., 2001; Steeg, 2003; Eccles and Welch, 2007). Metastasis suppressors are molecules that inhibit metastasis formation without affecting primary tumor growth (Table 1). Metastasis suppressor genes affect many steps of the metastatic process. Their specific regulation in this process is critical for the understanding of tumor metastasis. Here we review the discovery, molecular mechanisms and potential clinical applications of metastasis suppressor genes.

Discovery of metastasis suppressors

Metastasis suppressors were first identified using a microcell-mediated chromosome transfer (MMCT) approach. MMCT introduces chromosomes into intact recipient cells (Fournier and Ruddle, 1977). Human/mouse hybrid A9 donor cells carrying a single human chromosome containing potential metastasis suppressors were fused with recipient cells to generate microcell hybrids (Fournier and Ruddle, 1977). Spontaneous metastatic ability of these microcell hybrids was assayed in mouse models (Yoshida et al., 2000). Chromosomes 1, 6, 7, 8, 10, 11, 12, 16, and 17 were found to harbor metastasis suppressors (Ichikawa et al., 1991, 1992; Rinker-Schaeffer et al., 1994; You et al., 1995; Nihei et al., 1995; Miele et al., 1996, 1997 Phillips et al., 1996; Nihei et al., 1996; Chekmareva et al., 1997; Kuramochi et al., 1997; Matsuda et al., 1997; Luu et al., 1998; Mashimo et al., 1998; Seraj et al., 2000). Comparative genomic hybridization (cGH) and PCR were used to isolate the suppressor genes from these chromosomes. Breast cancer metastasis suppressor 1 (BRMS1) is one of the genes isolated by MMCT (Seraj et al., 2000). It was identified by the transfer of chromosome 11 into the highly metastatic breast cancer cell MDA-MB-435 using MMCT, and its subsequent differential display for the identification of this gene (Seraj et al., 2000). It has been shown that, in addition to breast cancer, BRMS1 suppresses metastasis in multiple tumor types including ovarian, bladder, melanoma and non-small cell lung carcinoma (Seraj et al., 2001; Shevde et al., 2002; Zhang et al., 2006; Smith et al., 2009). Yeast two-hybrid screen and co-immunoprecipitation demonstrated that BRMS1 interacts with multiple proteins including ARID4A and SUDS3, both of which are components of the SIN3 histone deacetylase chromatin remodeling complex.
Differential gene expression was further developed to isolate metastasis suppressors. Before the genome-wide detection of gene expression became available, differential colony hybridization was developed to compare gene expression from two different cell populations with differential metastatic potentials. NM23 was the first metastasis suppressor isolated using this method (Steeg et al., 1988). NM23 has eight family members but only NM23-H1 and NM23-H2 suppress metastasis in multiple tumor types (Lacombe et al., 2000). NM23 expression can serve as a potential prognostic marker for survival in breast, ovarian, melanoma, gastric, hepatocellular and non-small cell carcinoma (Hartsough and Steeg, 2000; Mao et al., 2001; Niu et al., 2002; Katakura et al., 2002; Wang et al., 2004; Guan-Zhen et al., 2007). NM23 has multiple functions including exonuclease, genomic stability maintenance, NDP and histidine kinases (Biggs et al., 1990; Freije et al., 1997; Ma et al., 2004; Kaetzel et al., 2009). It affects multiple cellular pathways including the MAPK pathway and the cytoskeleton-organizing pathway, which contribute to its metastasis-suppressing functions (Hartsough et al., 2002).

The development of genome-wide gene expression technologies enables the determination of gene expression profiling in tumor cells and tissues. Gene expressions in cell lines with differential metastatic capabilities were compared (Kang et al., 2003; Minn et al., 2005; Bos et al., 2009). These genes include both protein-coding genes and noncoding RNAs. MicroRNAs (miRNAs) are single-stranded noncoding RNAs of 21-23 nucleotides (He and Hannon, 2004; Pillai et al., 2007). They are a novel class of gene regulators that function by binding the 3' untranslated regions of target messenger RNAs leading to either suppression of their translation oracceleration of their degradation (He and Hannon, 2004; Pillai et al., 2007). miRNA expression was compared in the MDA-MB-231 cell and its metastatic variant cell derived from MDA-MB-231. Six miRNAs were found to have lower expression in metastatic derivative cells than in parent cells. Among these six miRNAs, miR-335 and miR-126 suppress metastasis without affecting primary tumor growth (Tavazoie et al., 2008). miR-335 targets multiple pathways including SOX4, MERTK, PTPRN2 and TNC, which contributes to its metastasis-suppressing functions (Tavazoie et al., 2008). miR-335 expression is also correlated with metastasis-free survival in clinical breast cancer (Tavazoie et al., 2008).

A functional genomics approach provides another method to isolate metastasis suppressors. The development of RNA interference (RNAi) technology allows for gene suppression in mammalian cells. Forward genetic screens using a genome-wide RNAi library in a mouse model enables the identification of metastasis suppressors without a priori knowledge of their functions. A genome-wide short hairpin RNA (shRNA) library was introduced into non-metastatic mouse mammary tumor 168FARN cells that were transplanted into mouse mammary fat pads (Gumireddy et al., 2009). The development of lung metastases serves as a positive selection system. Cells with knockdown of a metastasis suppressor will disseminate from mammary fat pad to secondary organs such as lung. These metastatic cells in lung were isolated for the retrieval of shRNA by PCR. This functional genomics screen identified Krüppel-like factor 17 (KLF17) as a novel metastasis suppressor (Gumireddy et al., 2009). Ectopic expression of KLF17 in highly metastatic 4T1 cells suppresses its metastatic potential without affecting the growth of primary tumor in a mouse model (Gumireddy et al., 2009). KLF17 belongs to a family of transcription factors with 17 members (Gumireddy and Huang, 2010). The suppression of KLF17 promotes tumor cell epithelial-mesenchymal transition (EMT) which leads to cell invasion and metastasis (Gumireddy et al., 2009). Microarray analysis identified transcription factor Id1 as a direct target of KLF17 and mediates its functions in metastasis.

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metastasis (Gumireddy et al., 2009). KLF17 expression is significantly downregulated and Id1 expression is upregulated in breast cancer metastasis, and the combination of KLF17 and Id1 expression may serve as a biomarker for breast cancer metastasis (Gumireddy et al., 2009).

A forward genetic screen in an in vitro model identified the metastasis suppressor GAS1 in melanoma (Gobeil et al., 2008). A genome-wide mouse RNAi library was introduced into poorly metastatic B16-F0 mouse melanoma cells, which were subsequently grown in 3D cell culture containing collagen and matrigel (Gobeil et al., 2008). Colony formation was used as a selection marker. shRNAs were retrieved from the cells that formed colonies in 3D culture. Knockdown of GAS1, one of the 22 candidate genes identified from the screen, promoted metastasis without affecting primary tumor growth (Gobeil et al., 2008). GAS1 suppresses metastasis by promoting apoptosis in disseminated cancer cells at secondary organs (Gobeil et al., 2008). GAS1 expression is downregulated in metastatic clinical samples (Gobeil et al., 2008).

The development of high-throughput sequencing and single nucleotide polymorphism (SNP) technologies allows for the detection of somatic mutations and genetic variations in clinical tumor samples at the genome-wide level. Next generation sequencing has identified potential candidate genes that play critical roles in metastasis including metastasis suppressor candidates. High-resolution SNP arrays were used to detect genetic variants in the primary tumor samples of colorectal cancer patients with or without liver metastasis (Ghadimi et al., 2006; Al-Mulla et al., 2006a; Sayagues et al., 2010). Primary tumors with known liver metastasis showed gain of chromosome 7p, 8q, 13q, and 20q, and loss of chromosome 1p, 8p, 9p, 14q, 17p and 22q. Genes that are located in the regions of chromosomal loss include MAP2K4, LLGL1, FBLN1, ELAC2, ALDH3A2, ALDH3A1, SHMT1, ARSA, WNT7B, TNFRSF13B, UPK3A, TYMP, RASD1, PEMT, and TOP3A. These candidate genes can potentially serve as metastasis suppressors and regulate the metastatic process in colorectal cancer.

Whole-genome sequencing was also used to identify mutations that are specific in clinical metastatic samples. Genome sequencing of a basal-like primary breast cancer, its matched brain metastases and normal tissues found two de novo mutations, SNED1 and FLNC, which are specifically for metastases (Ding et al., 2010). The functions of these two genes in the metastatic process have yet to be characterized. The development of low-cost sequencing technologies will enable larger numbers of tumor samples to be sequenced to validate these results and identify novel metastasis suppressors.

**Clinical applications of metastasis suppressors**

Although the impact of metastasis suppressors in current clinical practice is limited, metastasis suppressors can potentially serve as prognostic markers, therapeutic targets, and predictors for treatment response. NM23 expression has been found to be correlated with good prognosis in many tumor types. High NM23 expression has been found to be correlated with increased survival after cisplatin treatment (Bevilacqua et al., 1989; Hennessy et al., 1991; Royds et al., 1993; Tokunaga et al., 1993; Yamaguchi et al., 1994; Xerri et al., 1994; Toulas et al., 1996; Kawakubo et al., 1997; Charpin et al., 1998; Heimann et al., 1998; Müller et al., 1998; Ohno et al., 2000; Tas et al., 2002). For example, high NM23 expression is associated with overall survival and disease-free survival in breast cancer in several studies (Bevilacqua et al., 1989; Hennessy et al., 1991; Royds et al., 1993; Tokunaga et al., 1993; Toulas et al., 1996; Charpin et al., 1998; Heimann et al., 1998). The expression of KAI1, PEBP1 and RECK have also been shown to correlate with improved survival in multiple tumor types (Adachi et al., 1996, 1998; Huang et al., 1998; Sho et al., 1998; Muneyuki et al., 2001; Schindl et al., 2001; Hashida et al., 2003; Masui et al., 2003; Takeuchi et al., 2004; Takenaka et al., 2004; Al-Mulla et al., 2006b; Song et al., 2006; Wu et al., 2006; Chatterjee et al., 2008; Martinho et al., 2009; Xu et al., 2010; Zhang et al., 2012). High KAI1 or PEBP or RECK expression is associated with improved overall survival and disease-free survival in colorectal cancer (Hashida et al., 2003; Takeuchi et al., 2004; Al-Mulla et al., 2006b). The correlation between expression of other metastasis suppressors and good prognosis depends on tumor types. For example, high expression of CTGF is correlated with improved survival in colorectal cancer, non-small cell lung carcinoma and gallbladder cancer, but inversely correlated with survival in esophageal cancer and glioma (Xie et al., 2004; Lin et al., 2005; Chen et al., 2007; Alvarez et al., 2008; Zhou et al., 2009). These clinical observations suggest that the functions of some metastasis suppressor genes are cancer cell-type and cell-context dependent.

Metastasis suppressors can also potentially be used to determine which patients are more likely to respond to a particular treatment. It has been shown that patients with NM23 -positive ovarian cancer respond better to cisplatin than patients with NM23-negative tumors (Scambia et al., 1996). Similar observations were also found in patients with esophageal squamous cell carcinoma (Iizuka et al., 1999). NM23 expression is correlated with increased survival after cisplatin treatment following surgery (Iizuka et al., 1999). These studies demonstrate that the expression of metastasis suppressors can potentially be used to select patients for particular treatments.

Unlike tumor suppressors, most metastasis suppressors are not mutated in the majority of clinical tumor samples, but rather the expressions are downregulated. This observation raises the possibility that the activation of these metastasis suppressors can
potentially block metastasis and improve survival. It has been shown that the promoter region of NM23 contains glucocorticoid response elements that can elevate NM23 expression (Ouatas et al., 2002). Treatment of human breast cancer cell lines with dexamethasone medroxyprogesterone acetate (MPA) dramatically increases NM23 expression (Ouatas et al., 2003). Treatment of MPA in a mouse model significantly decreases pulmonary metastases (Palmieri et al., 2005). Clinical trials are underway to determine whether MPA can be used to improve survival in patients (Steeg and Theodorescu, 2007; Marshall et al., 2009a,b).

Studies of another metastasis suppressor, RhoGDI2, found that RhoGDI2 suppresses endothelin 1 (ET1), a vasoconstrictor whose expression is correlated with higher clinical stage in bladder cancer (Titus et al., 2005). Administration of atrasentan, an ET1 antagonist currently used in a clinical trial, significantly reduced pulmonary metastasis in a mouse model (Titus et al., 2005). These results indicate that metastasis suppressors or their downstream targets can serve as potential therapeutic targets in cancer patients.

**Perspectives**

Considering the complexity of the metastatic process, it is likely that additional metastasis suppressor genes have yet to be isolated. Genome-wide study of gene expression, genetic variants, somatic mutations, alterations in epigenetic regulation in the tumor and genetic background of patients in clinical samples and laboratory models will identify novel metastasis suppressor candidate genes. Functional characterization of these candidate genes is required to differentiate *bona fide* metastatic suppressors without affecting primary tumor growth. Cross-talk between primary tumor, metastatic cells and tumor microenvironment is critical for the development of metastases. Systemic factors that mediate metastases formation and growth are also important in the metastatic process. These are understudied areas. It is possible that some metastasis suppressors function in the communication between environment and metastatic cells.

The applications of metastasis suppressors in clinical patient care are currently limited. With the identification of novel metastasis suppressors, it is conceivable that they can serve as more robust prognostic markers and as predictors for the selection of patients who respond well to particular treatment. Some of the metastasis suppressors should be able to serve as therapeutic targets based on the effects they have on tumor cells and the pathways in which they are involved. It is also possible that better therapeutic effects will be achieved when drugs that target metastasis suppressors are used in combination with other treatments. The discovery of metastasis suppressor genes has transformed metastasis research and will continue to contribute to the transformation of metastasis into a curable or manageable disease.

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