Commentary

New Insights into the Tumor Metastatic Process Revealed by Gene Expression Profiling

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Gene expression profiling holds promise for cancer diagnosis, the identification of prognostic and predictive markers, and the discovery of fundamental insights into cancer processes such as metastasis. Despite increasing numbers of papers devoted to gene expression profiling, interpretation of the data has been subject to many variables and our understanding remains incomplete. In this issue of *The American Journal of Pathology*, Montel et al¹ report an extensive gene expression profiling analysis using xenografts of MDA-MB-435 human breast carcinoma cells. The report, because of the relative simplicity of the model system as compared to human tumors, provides several clear results for further consideration.

The model system consisted of two clonal sublines of MDA-MB-435 breast carcinoma cells, designated NM-2C5 and CL 16, which were of low and high tumor metastatic potential respectively to both the lungs and lymph nodes. The CL 16 line was the product of five rounds of serial orthotopic injection of a high metastatic subline, and re-culture of metastatic lesions. Both sublines were labeled to visualize metastases by fluorescence. The MDA-MB-435 cell line has been reported to be breast in origin, with phenotypic heterogeneity on microarray analysis.² Microarrays were conducted comparing primary tumors from each line to lymph node metastases of the same subline, lung metastases of the same subline, and primary tumors of the other subline. Data were obtained using tumors from SCID mice as a training set and validated with data from the same lines injected into nude mice as a test set. The authors also performed microarrays on *in vitro* cultures of the sublines. A proportion of the differentially expressed genes were confirmed by RT-PCR and by protein methods.

General Conclusions

The most obvious conclusion matches that of many recent papers in the literature, that the expression profile of

the metastases closely resembled the primary tumors. This is consistent with previous reports using limited human breast cancer tumor cohorts^{3–5} and has been used to question the evolution of metastases. However, the depth of the data in this paper permits several other fascinating conclusions to be drawn.

Most emphatically, the gene expression signature of the low metastatic subline was distinct from that of the high metastatic subline. Metastases of the low metastatic subline closely resembled the profile of the low metastatic primary tumor, and did not “switch over” to the high metastatic gene expression profile. Thus, heterogeneity exists in this cell line/tumor, observable through the subcloning. Such heterogeneity may exist in human tumors, but may not be apparent as the signature reflects the majority of the tumor cells. The low metastatic subline is capable of spawning metastases, albeit at a lower rate, and its gene expression profile is therefore of significant interest. Thus, not all relevant metastatic signatures may be obtained from ground up tumors.

The next conclusion should not be startling. The gene expression profile of the sublines as *in vitro* cultures was distinct from the same sublines as primary tumors. Very distinct. While the primary tumors are a mixture of tumor cells, stromal cells, endothelial cells, etc, the contribution of these cells to the observed gene expression profiles should be minimal as they are murine in origin. Thus, the differences are thought to reflect changes in the tumor cells in response to the *in vivo* microenvironment. Further *in situ* hybridization and immunohistochemical experiments will refine this conclusion.

Comparable numbers of genes were “turned on” and “turned off” in the more highly metastatic tissues. We think about metastasis as the acquisition of traits, but the data remind us to pay equal attention to the loss of growth and differentiation-controlling genes. The metastasis suppressor genes are prime examples of genes lost in metastasis.^{6,7}

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Table 1. Selected Genes of Potential Interest to Metastasis Research from Microarray Data

Gene	Up-regulated in	Compared to	Function	Reference
Kinesin 4A	NM Pri.	M Pri.	Chromosomal abnormalities: alignment and segregation of chromosomes	8
MAX interacting protein 1	NM Pri	NM Lung	Potential suppressor: antagonist of c-Myc	9–11
Regulator of G-protein signaling 10	NM Pri.	M Pri.	Signaling: reduces heterotrimeric G-protein signaling, activates G α i	17
Plectin 1	NM Pri.	NM LN	Signaling: regulation of protein kinase C activity	18
TYRO3	NM Pri.	NM Lung	Signaling: receptor tyrosine kinase	22
Arginase II	NM Lung	NM Pri.	Regulation of nitric oxide synthesis	23
Rab 27	NM LN	NM Pri.	Vesicular fusion, trafficking	24
Rab 38	M LN	M Pri.	Vesicular fusion, trafficking	25
VAMP associated protein A	M Lung	M Pri.	Membrane fusion, trafficking	26
Syntaxin 7	M LN	M Pri.	Vesicle transport	27
Synaptosomal-associated protein	NM Pri.	NM Lung	Membrane fusion, trafficking	28
Malate dehydrogenase 1	NM LN	NM Pri.	TCA cycle	
Isocitrate dehydrogenase 3	M LN	M Pri.	TCA cycle	

NM, non- or poorly metastatic; M, metastatic; Pri., primary tumor; LN, lymph node metastasis; Lung, lung metastasis.

Viva la Difference!

Despite all of the attention given to the overall similarity of primary tumors and metastases, differences existed in these experiments as well as in other reports. These differences are fascinating and serve as leads for functional contributions, testable in transfection experiments. Many of the differentially expressed genes fall into the category of “usual suspects” for metastasis. These include the overexpression of osteopontin and extracellular matrix genes in the more metastatic tissues, and the loss of thrombospondin 1 and members of the Nm23 family. Other genes are unexpected and, if validated functionally, could shed new light on the mechanisms of metastasis. Table 1 lists several of the genes I found in the report.¹ Some highlights are described below.

Chromokinesin 4A

We have known for years that metastatic tumors are genetically unstable, but why? Chromokinesin 4A is involved in the faithful segregation of chromosomes in mitosis,⁸ which may prevent aneuploidy. It's more highly expressed in the nonmetastatic than the metastatic primary tumors.

Max Interacting Protein 1, Mxi1

This gene appeared twice, more highly expressed in the nonmetastatic primary tumor than either its lung or lymph node metastases. Mxi1 is an antagonist of c-Myc. It appears to have tumor suppressor activity in both prostate and glioma model systems, possibly through the regulation of cyclin B expression.^{9–11}

Regulator of G Protein Signaling 10 (RGS10)

Certain heterotrimeric G proteins mediate signaling pathways important to metastasis including chemokines,¹² the Kiss-1 metastasis suppressor encoded peptide^{13–15} and the physiology of lysophosphatidic acid;¹⁶ however the intricacies of the pathways have not been fully elucidated. RGS10 was down-regulated in the metastatic primary tumor as compared to the nonmetastatic primary tumor; it functions to limit G protein signaling by activating the inhibitory (G α i) component.¹⁷

Plectin 1 (PLEC1)

This is one of those renaissance proteins that performs multiple tasks. Originally identified as a component of intermediate filaments, PLEC1 mutations were also identified as the cause of muscular dystrophy with epidermolysis bullosa simplex. Recently, plectin was reported to associate with receptor for activated C kinase (RACK1) and protein kinase C δ , inhibiting kinase activity.¹⁸ Plectin 1 was overexpressed in nonmetastatic primary tumor as compared to its lymph node metastasis.

Rabs 27 and 38

The intracellular exocytic and endocytic pathways are regulated by the spatial distribution of organelles and an elaborate vesicle transport system. The importance of these pathways to cancer growth and metastasis is just beginning to emerge. Rab proteins are small GTPases functioning in various stages of vesicular fusion and trafficking. Rab27 regulates the exocytosis of cell-type specific storage organelles, such as melanosomes in mela-

nocytes and lytic granules in cytotoxic T lymphocytes.¹⁹ Rab38 may play a similar role, as it has been linked to the production of tyrosinase in melanocytes.²⁰ These genes were identified in three pair-wise sets, in each case overexpressed in the more metastatic tissue.

Other Targets Involved in the Exocytic and Endocytic Processes

Vesicle-associated membrane protein-associated protein A (VAPA) interacts with Vesicle-associated membrane proteins (VAMPs), which are part of the SNARE family that mediate the fusion of organelle membranes. Similarly, syntaxin 7 mediates vesicle attachment to lysosomes. Both genes were overexpressed in the more metastatic tissue, similar to the Rabs. In contrast, synaptosomal-associated protein 23 (SNAP23) binds syntaxins and VAMPs and shows the opposite expression pattern.

Lessons from Tissues

A second example of this type of analysis was published by Hao et al²¹ and includes gene expression analysis of nine sets of matched breast cancer primary tumors and lymph node metastases. Microarray analysis of the nine sets of tissues failed to distinguish primary tumors from lymph node metastases when all genes in the microarray were used for multidimensional scaling (MDS). The authors then reasoned that multiple pathways lead to the metastatic phenotype and identified 280 genes exhibiting significant differential expression in $\geq 3/9$ pairs. Several genes or gene families from this differentially expressed list matched the trends reported by Montel et al, including thrombospondin 1, integrin $\beta 1$, the cadherin family and the Nm23 family. Of the genes described above from Montel et al, another Rab family member (Rab2) was overexpressed in the lymph node metastases from the Hao et al database; the Max interacting protein 1 gene from Montel et al displayed the opposite expression pattern as the Max gene in Hao et al. These data, if confirmed in functional experiments, suggest that non-universal but important gene expression differences may exist between primary tumors and metastatic lesions.

Opportunities

The elegance of the MDA-MB-435 model system described by Montel et al¹ will certainly stimulate further analysis. Using laser capture microdissection and amplification, it would be fascinating to compare smaller micrometastases versus larger macroscopic metastases. The smaller lesions may identify gene expression targets that are therapeutically tractable against minimal residual disease.

Can this system also model tumor dormancy? It is not clear when a cell line is low- to non-metastatic versus when it is "dormant." To what extent has the low metastatic subline described herein produced micrometastases at the endpoints described, and do they colonize

given more time? If so, how does the gene expression of early versus late metastases compare? This question is of enormous importance, as breast and other cancers recur 5 to 30 years after initial treatment.

The data could be extended to include the pre- and post-treatment gene expression profiles of the response of low and highly metastatic tumors to conventional and developmental therapeutics. What genes accompany resistance in each subline? The relevance of the data published by Montel et al as well as these suggested experiments will be assessed in functional assays and by comparison to human tumor cohorts.

References

1. Montel V, Huang T-Y, Mose E, Pestonjamas K, Tarin D: Expression profiling of primary tumors and matched lymphatic and lung metastases in a xenogeneic breast cancer model. *Am J Pathol* 2005, 166:1565–1579
2. Sellappan S, Grijalva R, Zhou X, Yang W, Eli MB, Mills G, Yu D: Lineage infidelity of MDA-MB-435 cells: Expression of melanocyte proteins in a breast cancer cell line. *Cancer Res* 2004, 64:3479–3485
3. Perou C, Serlie T, Eisen M, Rijn Mvd, Jeffrey S, Rees C, Pollack J, Ross D, Johnson H, Akslen L, Fluge O, Pergamenschikov A, Williams C, Zhu S, Lenning P, Borresen-Dale A, Brown P, Botstein D: Molecular portraits of human breast tumors. *Nature* 2000, 406:747–752
4. Weigelt B, Glas A, Wessels L, Witteveen A, Peterse J, Veer LV: Gene expression profiles of primary breast tumors maintained in distant metastases. *Proc Natl Acad Sci USA* 2003, 100:15901–15905
5. Adib T, Henderson S, Perrett C, Hewitt D, Bourmpoulia D, Ledermann J, Boshoff C: Predicting biomarkers for ovarian cancer using gene-expression microarrays. *Br J Cancer* 2004, 90:686–692
6. Steeg P: Metastasis suppressors alter the signal transduction of cancer cells. *Nature Cancer Rev* 2003, 3:55–63
7. Yoshida B, Sokoloff M, Welch D, Rinker-Schaeffer C: Metastasis-suppressor genes: a review and perspective on an emerging field. *J Natl Cancer Inst* 2000, 92:1717–1730
8. Mazumdar M, Sundareshan S, Misteli T: Human chromokinesin KIF4A functions in chromosome condensation and segregation. *J Cell Biol* 2004, 166:613–620
9. Taj M, Tawil R, Engstrom L, Zeng Z, Hwang C, Sanda M, Wechs D: Mxi1, a Myc antagonist, suppresses proliferation of DU145 human prostate cells. *Prostate* 2001, 47:194–204
10. Manni I, Tunici P, Cirenei N, Albarosa R, Colombo B, Roz L, Sacchi A, Piaggio G, Finocchiaro G: Mxi1 inhibits the proliferation of U87 glioma cells through down regulation of cyclin B1 gene expression. *Br J Cancer* 2002, 86:477–484
11. Zervos A, Gyuris J, Brent R: Mxi1, a protein that specifically interacts with Max to bind Myc:Max recognition sites. *Cell* 1993, 72:223–232
12. Arya M, Patel H, Williamson M: Chemokines: key players in cancer. *Curr Med Res Opin* 2003, 19:557–564
13. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden J, LePoul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann S, Vassart G, Parmentier M: The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GRP54. *J Biol Chem* 2001, 276:34631–34636
14. Muir A, Chamberlain L, Elshourbagy N, Machalovich D, Moore D, Calamari A, Szekeres P, Sarau H, Chambers J, Murdock P, Steplewski K, Shabon U, Miller J, Middleton S, Darker J, Larminie C, Wilson S, Bergsma D, Emerson P, Faull R, Philpott K, Harrison D: AXOR12, a novel human G protein-coupled receptor, activated by the peptide Kiss-1. *J Biol Chem* 2001, 276:28969–28975
15. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M: Metastasis suppressor Kiss-1 encodes a peptide ligand of a G-protein-coupled receptor. *Nature* 2001, 411:613–617

16. Mills G, Moolenaar W: The emerging role of lysophosphatidic acid in cancer. *Nat Rev Cancer* 2003, 3:582–581
17. Hunt T, Fields T, Casey P, Peralta E: RGS10 is a selective activator of G α i GTPase activity. *Nature* 1996, 383:175–177
18. Osmanagic-Meyers S, Wiche G: Plectin-RACK1 (receptor for activated C kinase 1) scaffolding: a novel mechanism to regulate protein kinase C activity. *J Biol Chem* 2004, 279:18701–18710
19. Izumi T, Gomi H, Kasai K, Mizutani S, Torii S: Cell Structure and Function. 28 The roles of Rab27 and its effectors in the regulated secretory pathways. *Cell Struct Funct* 2003, 28:465–474
20. Loftus S, Larson D, Baxter L, Antonellis A, Chen Y, Wu X, Jiang Y, Bittner M, Hammer J, Pavan W: Mutation of melanosome protein RAB38 in chocolate mice. *Proc Natl Acad Sci USA* 2002, 99:4471–4476
21. Hao X, Sun B, Hu L, Lahdesmaki H, Dunmire V, Feng Y, Zhang S-W, Wang H, Wu C, Wang H, Fuller G, Symmans W, Shmulevich I, Zhang W: Differential gene and protein expression in primary breast malignancies and their lymph node metastases as revealed by combined cDNA microarray and tissue microarray analysis. *Cancer* 2004, 100:1110–1122
22. Liao X, Zhou R, Gilbert D, Copeland N, Jenkins N: Receptor tyrosine kinase gene Tyro3 maps to mouse chromosome 2, closely linked to Ltk. *Mammalian Genome* 1996, 7:395–396
23. Vockley J, Jenkinson C, Shukla H, Kern R, Grody W, Cederbaum S: Cloning and characterization of the human type II arginase gene. *Genomics* 1996, 38:118–123
24. Nagata K, Itoh H, Katada T, Takenaka K, Ui M, Kaziro Y, Nozawa Y: Purification, identification and characterization of two GTP-binding proteins with molecular weights of 25,000 and 21,000 in human platelet cytosol: one is the rap1/smg21/krev-1 protein and the other is a novel GTP-binding protein. *J Biol Chem* 1989, 264:17000–17005
25. Jager D, Stockert E, Jager E, Gure A, Scanlan M, Knuth A, Old L, Chen Y-T: Serological cloning of a melanocyte rab guanosine 5-prime-triphosphate-binding protein and a chromosome condensation protein from a melanoma complementary DNA library. *Cancer Res* 2000, 60:3584–3591
26. Weir M, Klip A, Trimble W: Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds VAMP. *Biochem J* 1998, 333:247–251
27. Wang H, Frelin L, Pevsner J: Human syntaxin 7: a Pep12p/Vps6p homologue implicated in vesicle trafficking to lysosomes. *Gene* 1997, 199:39–48
28. Ravichandran V, Chawla A, Roche P: Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-cueral tissues. *J Biol Chem* 1996, 271:13300–13303