MAPK, NFκB, and VEGF signaling pathways regulate breast cancer liver metastasis

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ABSTRACT

In this study, we investigated the molecular pathways regulating breast cancer liver metastasis. We identified 48 differentially expressed genes (4 upregulated and 44 downregulated) by analyzing microarray dataset GSE62598 from Gene Expression Omnibus (GEO). We constructed a genetic interaction network with 84 nodes and 237 edges using the String consortium database. The network was reliably robust with a clustering coefficient (cc) of 0.598 and protein-protein interaction (PPI) enrichment p value of zero. Using the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases, we identified MAPK, NFκB and VEGF signaling pathways as the most critical pathways regulating breast cancer liver metastasis. These results indicate that the distinct breast cancer metastatic stages, including dissemination from the primary breast tumor, transit through the vasculature, and survival and proliferation in the liver, are regulated by the MAPK, NFκB, and VEGF signaling pathways.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer globally and is the leading cause of cancer-related deaths among women [1]. In the United States, more than 240,000 newly diagnosed breast cancer cases and 40,000 deaths were reported in 2016 [2]. Liver metastasis is reported in 15% of newly diagnosed breast cancer patients [3, 4]. Breast cancer liver metastasis is associated with very poor prognosis and has a survival time of only 4-8 months, if untreated [5]. Introduction of new therapies in the last decade has resulted in 1-2% yearly decrease in mortality rates [6]. However, breast cancer patients with liver metastasis still are associated with very poor outcomes [7].

Metastatic disease is a complex, multistage process that involves detachment of breast cancer cells from the primary tumor, which then travel through the blood or lymphatic system and finally survive and proliferate in the liver. Given the complex multistep process, liver metastasis involves a sophisticated network of molecular events. However, the molecular mechanisms associated with breast cancer metastasis to the liver are unclear, and their understanding is essential for developing more effective therapies. In this study, therefore, we generated a genetic interaction network using microarray gene expression data from breast cancer liver metastases and explored the molecular mechanisms involved using bioinformatic analyses.

RESULTS

Forty-eight genes are differentially expressed in metastatic breast tumor cells

Table 1 lists the differentially expressed genes with a fold change ≥2 and false discovery rate ≤ 5%. There were 48 differentially expressed genes that were distinctly upregulated (4 genes) or downregulated (44 genes) in metastatic tumor cells than in normal parental cells. Figure 1 shows the heat map of the differentially expressed genes.
| Gene ID     | Gene Name | Fold Change | Gene regulation |
|------------|-----------|-------------|-----------------|
| A_52_P618173 | Limch1    | 2.290749902 | Up              |
| A_52_P418791 | Rbp1      | 2.424147188 | Up              |
| A_51_P423484 | Rbp1      | 2.165856946 | Up              |
| A_52_P299915 | Map2k6    | 2.176087369 | Up              |
| A_51_P102538 | Otop1     | 0.336723951 | Down            |
| A_51_P289341 | Fermt1    | 0.317362329 | Down            |
| A_52_P452667 | Prom2     | 0.285970233 | Down            |
| A_51_P333923 | Tspan1    | 0.315241505 | Down            |
| A_51_P167489 | Lama3      | 0.41612039  | Down            |
| A_51_P177242 | Unc13b    | 0.418318499 | Down            |
| A_52_P88091  | Dsg2      | 0.403969687 | Down            |
| A_51_P233153 | Cadps2    | 0.298078637 | Down            |
| A_51_P196207 | Capsl     | 0.388252581 | Down            |
| A_52_P79821  | Esrp1     | 0.26893644  | Down            |
| A_52_P559779 | Dsg2      | 0.347328438 | Down            |
| A_51_P493987 | Moxd1     | 0.417459194 | Down            |
| A_52_P87757  | Il24      | 0.336785971 | Down            |
| A_52_P134455 | Fermt1    | 0.367135842 | Down            |
| A_51_P356055 | Grp       | 0.449573589 | Down            |
| A_51_P353252 | Mal2      | 0.291415896 | Down            |
| A_51_P187602 | Serpinb5  | 0.3120555   | Down            |
| A_52_P638605 | Ap1m2     | 0.436913739 | Down            |
| A_51_P105879 | Myo5b     | 0.486596961 | Down            |
| A_52_P405945 | Prl3d2    | 0.483474132 | Down            |
| A_51_P401517 | Il24      | 0.483144818 | Down            |
| A_52_P252931 | Dsc2      | 0.491809463 | Down            |
| A_52_P468068 | Tchh      | 0.490774711 | Down            |
| A_51_P322115 | Htr5b     | 0.372641522 | Down            |
| A_52_P286350 | Sh2d1b1   | 0.471867312 | Down            |
| A_52_P487686 | BC100530  | 0.483518325 | Down            |
| A_51_P489488 | Pde4dip   | 0.487698119 | Down            |
| A_51_P179293 | 2310002L13Rik | 0.382311761 | Down            |
| A_51_P322090 | Ovol2     | 0.489037358 | Down            |
| A_52_P661412 | Adora1    | 0.485167002 | Down            |
| A_52_P683580 | Tbc1d9    | 0.471654273 | Down            |
| A_51_P206475 | Lce1i     | 0.476512201 | Down            |
| A_51_P496540 | Sh2d1b1   | 0.488430246 | Down            |
| Gene ID          | Gene Name | Fold Change  | Gene regulation |
|-----------------|-----------|--------------|-----------------|
| A_52_P601757    | Dsg2      | 0.414988774  | Down            |
| A_51_P496253    | Slc6a4    | 0.464974691  | Down            |
| A_51_P438283    | Il1a      | 0.497937489  | Down            |
| A_51_P455620    | Fam167a   | 0.45781262   | Down            |
| A_51_P332309    | Eomes     | 0.434829918  | Down            |
| A_51_P225827    | Ovol1     | 0.474676527  | Down            |
| A_51_P338878    | P2ry12    | 0.424196491  | Down            |
| A_52_P373982    | Grhl2     | 0.481346604  | Down            |
| A_52_P642488    | Kcnk1     | 0.43461204   | Down            |
| A_51_P303079    | Tmem54    | 0.492962995  | Down            |
| A_51_P362328    | Grhl2     | 0.469572322  | Down            |

Abbreviation: SAM, Significance Analysis Microarray

Figure 1: Heatmap visualization of the differently expressed genes identified by Significant Analysis of Microarray (SAM) in metastatic tumor cells (GSM1529777, GSM1529778, GSM1529779) versus 4T1 parental cells (GSM1529768, GSM1529769, GSM1529770). Red represents up-regulated genes, while green represents down-regulated genes.
A genetic interaction network based on the
differently expressed genes

A genetic interaction network was constructed from the 48 differentially expressed genes using the String platform future analysis (Figure 2). The interaction network consisted of 84 nodes and 237 edges. The average node degree was 5.64. The network was reliably robust with a clustering coefficient (cc) of 0.598 and protein-protein interaction (PPI) enrichment p value of zero.

GO analysis of the differently expressed genes

Molecular function analysis by the GO consortium database revealed that most of the differently expressed genes regulated protein binding and kinase activity (Table 2). Besides, the major biological processes associated with the liver metastases were positive regulation of cell communication, MAPK cascade, signaling, and protein kinase activity (Table 3).

Signaling pathways involved in breast cancer liver metastasis

Table 4 shows the signaling pathways involved in breast cancer liver metastases by the KEGG database. The major signaling pathways included the MAPK, NF-kappa B and VEGF signaling pathways that maybe critical for the distinct pathological stages of liver metastasis.

DISCUSSION

Breast cancer liver metastasis is a complex process that includes tumor cell dissemination from the primary tumor, transit through the blood or lymphatic system, and proliferation in liver. Underlying this complex multistep process is a sophisticated network of molecular events. In

Figure 2: Genetic interaction network associated with breast cancer liver metastases basing on String platform. In this figure, each circle represents a gene (node) and each connection represents a direct or indirect connection (edge). Line color indicates the type of interaction evidence and line thickness indicates the strength of data support.
this study, we generated, for the first time, a comprehensive genetic interaction network from the microarray gene expression profile to identify the molecular mechanisms involved in breast cancer liver metastases. The results suggested that MAPK, NF-κB and VEGF signaling pathways are significantly associated with distinct stages of breast cancer liver metastasis.

Dissemination of carcinoma cells is the initial step of the metastasis, which is initiated by epithelial-mesenchymal transition (EMT) program during which tumor cells acquire mesenchymal features and lose epithelial properties [8, 9]. The complex molecular events during EMT are initiated and controlled by signaling pathways that respond to extracellular cues. The transforming growth factor-β (TGF-β) signaling family plays a predominant role in EMT [10]. Moreover, the MAPK signaling pathway is required for the initiation of TGF-β induced EMT [11, 12]. In addition to TGF-β family proteins, tyrosine kinase receptors (RTKs) play a key role in the trans-differentiation process, further highlighting the importance of MAPK signaling [13]. MAPK pathway inhibitors have been used clinically for many cancers, including breast cancer [14]. In addition, NFκB is an important regulator of the expression of various proteins involved in the immune response [15].

After successfully disassociating from the primary tumor, metastatic carcinoma cells traverse the blood or lymphatic system, during which they interact with several cell types including platelets, neutrophils, monocytes, macrophages, and endothelial cells [16]. The circulating tumor cells also interact with platelets

| GO ID          | Molecular Function                                               | Observed Gene Count | FDR      |
|---------------|-----------------------------------------------------------------|---------------------|----------|
| GO.0004702    | receptor signaling protein serine/threonine kinase activity     | 15                  | 3.13E-21 |
| GO.0005515    | protein binding                                                 | 7                   | 2.03E-05 |
| GO.0004708    | MAP kinase kinase activity                                       | 41                  | 2.41E-05 |
| GO.0017137    | Rab GTPase binding                                              | 5                   | 2.74E-05 |
| GO.0031489    | myosin V binding                                                | 6                   | 0.000307 |
| GO.0017022    | myosin binding                                                  | 4                   | 0.000381 |
| GO.0004709    | MAP kinase kinase kinase activity                                | 5                   | 0.000518 |
| GO.0005488    | binding                                                         | 4                   | 0.00169  |
| GO.0017075    | syntaxin-1 binding                                              | 59                  | 0.00354  |
| GO.0004707    | MAP kinase activity                                             | 3                   | 0.00402  |
| GO.0004674    | protein serine/threonine kinase activity                        | 3                   | 0.00363  |
| GO.0004946    | bombesin receptor activity                                      | 9                   | 0.0113   |
| GO.0005102    | receptor binding                                                | 2                   | 0.0128   |
| GO.0004908    | interleukin-1 receptor activity                                 | 14                  | 0.018    |
| GO.0019905    | syntaxin binding                                                | 2                   | 0.0215   |
| GO.0019899    | enzyme binding                                                  | 4                   | 0.0253   |
| GO.0004871    | signal transducer activity                                      | 15                  | 0.032    |
| GO.0005179    | hormone activity                                                | 16                  | 0.0377   |
| GO.0060089    | molecular transducer activity                                   | 4                   | 0.0377   |
| GO.0086083    | cell adhesive protein binding involved in bundle of His cell-    | 17                  | 0.0377   |

Abbreviations: FDR, false discovery rate; GO, Gene Ontology.
high platelet counts are associated with poor prognosis in carcinomas [18]. Recent studies have revealed that platelets alter the fate of circulating cancer cells [19]. Platelet-tumor cell contacts and platelet-derived TGF-β synergistically activate the TGF-β/Smad and NFκB pathways in cancer cells enabling their transition to an invasive mesenchymal-like phenotype, thereby enhancing metastasis [20]. Inhibition of NFκB signaling in cancer cells or ablation of TGF-β1 expression in platelets protects against lung metastasis in vivo [20].

In the liver, a pre-metastatic niche is established by VEGFR+ bone marrow progenitors before the arrival of tumor cells [21]. In fact, the initial events during the development of metastasis are VEGF-dependent [22]. Once the metastatic cancer cells survive in the new environment, they undergo colonization before the onset of the final process of malignancy. In general, a tumor requires angiogenesis to grow beyond 1-2 mm in size. In the initial pre-vascular phase, the size of the tumor does not exceed a few millimeters, but, neovascularization results in rapid growth of the tumor. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis, which stimulates endothelial proliferation and migration, inhibits endothelial apoptosis, and increases vascular permeability and vasodilatation [23]. VEGF-targeting therapy has shown significant benefits in the treatment of metastatic breast cancer [24, 25]. In conclusion, based on the genetic interaction network, we identified MAPK, NF-kappa B and VEGF signaling pathways as key regulators of breast cancer liver metastasis.

Table 3: Biological process analysis of the genetic interaction network associated with liver-aggressive explant in terms of Gene Ontology (GO)

| GO ID    | Biological Process                                      | Observed Gene Count | FDR       |
|---------|---------------------------------------------------------|---------------------|-----------|
| GO.0051046 | regulation of secretion                                | 21                  | 5.45E-10  |
| GO.0080134 | regulation of response to stress                       | 28                  | 6.97E-10  |
| GO.1903530 | regulation of secretion by cell                        | 19                  | 4.53E-09  |
| GO.0051047 | positive regulation of secretion                        | 15                  | 8.72E-09  |
| GO.0032101 | regulation of response to external stimulus             | 20                  | 1.24E-07  |
| GO.0032879 | regulation of localization                              | 31                  | 1.24E-07  |
| GO.0051049 | regulation of transport                                 | 27                  | 1.24E-07  |
| GO.0051050 | positive regulation of transport                        | 20                  | 1.24E-07  |
| GO.0031347 | regulation of defense response                          | 18                  | 3.95E-07  |
| GO.0010647 | positive regulation of cell communication                | 25                  | 4.18E-07  |
| GO.0060341 | regulation of cellular localization                     | 22                  | 4.18E-07  |
| GO.0043410 | positive regulation of MAPK cascade                     | 14                  | 8.81E-07  |
| GO.0014047 | glutamate secretion                                     | 6                   | 1.17E-06  |
| GO.0050690 | regulation of defense response to virus by virus        | 6                   | 1.38E-06  |
| GO.0023056 | positive regulation of signaling                        | 23                  | 1.79E-06  |
| GO.0051650 | establishment of vesicle localization                   | 10                  | 2.00E-06  |
| GO.0046717 | acid secretion                                          | 7                   | 3.36E-06  |
| GO.0001934 | positive regulation of protein phosphorylation          | 17                  | 5.02E-06  |
| GO.0016079 | synaptic vesicle exocytosis                              | 37                  | 3.10E-13  |
| GO.0045860 | positive regulation of protein kinase activity          | 11                  | 3.55E-13  |

Abbreviations: FDR, false discovery rate; GO, Gene Ontology; MAPK: mitogen-activated protein kinase.
Table 4: Signaling pathway analysis of the genetic interaction network associated with liver-aggressive explant in terms of Gene Ontology (GO)

| Pathway ID | Signaling pathway                                         | Observed Gene Count | FDR            |
|------------|----------------------------------------------------------|---------------------|----------------|
| 4010       | MAPK signaling pathway                                    | 16                  | 1.42E-12       |
| 4668       | TNF signaling pathway                                     | 9                   | 7.29E-08       |
| 5014       | Amyotrophic lateral sclerosis (ALS)                       | 7                   | 1.26E-07       |
| 4750       | Inflammatory mediator regulation of TRP channels          | 8                   | 3.45E-07       |
| 4380       | Osteoclast differentiation                                | 8                   | 1.45E-06       |
| 5140       | Leishmaniasis                                             | 6                   | 1.24E-05       |
| 4721       | Synaptic vesicle cycle                                    | 5                   | 0.000104       |
| 4664       | Fc epsilon RI signaling pathway                           | 5                   | 0.000156       |
| 4660       | T cell receptor signaling pathway                          | 5                   | 0.000787       |
| 5146       | Amoebiasis                                                | 5                   | 0.000993       |
| 4060       | Cytokine-cytokine receptor interaction                    | 7                   | 0.00133        |
| 4722       | Neurotrophin signaling pathway                            | 5                   | 0.00145        |
| 5160       | Hepatitis C                                               | 5                   | 0.00206        |
| 4015       | Rap1 signaling pathway                                    | 6                   | 0.00207        |
| 4911       | Insulin secretion                                         | 4                   | 0.00355        |
| 4728       | Dopaminergic synapse                                       | 4                   | 0.0148         |
| 5131       | Shigellosis                                               | 3                   | 0.0148         |
| 4370       | VEGF signaling pathway                                    | 3                   | 0.0155         |
| 5162       | Measles                                                   | 4                   | 0.0162         |
| 5120       | Epithelial cell signaling in Helicobacter pylori infection| 3                   | 0.0194         |
| 5222       | Small cell lung cancer                                    | 3                   | 0.0351         |
| 4064       | NF-kappa B signaling pathway                              | 3                   | 0.0384         |
| 5168       | Herpes simplex infection                                  | 4                   | 0.0384         |
| 4723       | Retrograde endocannabinoid signaling                      | 3                   | 0.0473         |

Abbreviations: FDR, false discovery rate; GO, Gene Ontology.

**MATERIALS AND METHODS**

**Microarray dataset resources**

Microarray dataset with the accession number GSE62598 was downloaded from Gene Expression Omnibus (GEO). In this study, the authors examined if the propensity of breast cancer cells to metastasize to liver was associated with distinct patterns of immune cell infiltration [26]. Total RNA was extracted from 4T1 parental and individual metastatic sub-populations. The mRNA array was performed on Agilent-014868 Whole Mouse Genome Microarray 4×44k G4122F platform.

**Analysis of differentially expressed genes**

The gene expression profiles of metastatic tumor cells versus disseminated tumor cells were normalized by log_{10} transformation after normalization. Then, Significance Analysis of Microarrays software (SAM, http://statweb.stanford.edu/~tibs/SAM/) was used to produce a cluster of up- or down-regulated genes [27].

**Genetic interaction network construction**

Genetic interaction network was constructed using the String consortium database (http://string-db.org/). In addition, to identify the pathways involved Gene Ontology consortium
(GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) functional enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/).

Statistical analysis

According to a previous publication [28], gene expression was considered significant if the threshold of false discovery rate (FDR) ≤ 5% and fold change ≥ 2. For GO and KEGG enrichment analysis, biological process, molecular function and signaling pathways, $p ≤ 5\%$ was considered significant.

Author contributions

All authors contributed towards data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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