

Maintenance of Head and Neck Tumor Gene Expression Profiles upon Lymph Node Metastasis

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Abstract

Spread of cancer and development of solid metastases at distant sites is the main cause of cancer-related deaths. To understand and treat metastases, it is important to determine at which stages the most pivotal steps for development of metastases occur. In head and neck squamous cell carcinoma (HNSCC), metastasis nearly always occurs first in local lymph nodes before development of distant metastasis. Here, we have investigated gene expression patterns in HNSCC lymph node metastases using DNA microarrays. Several types of analyses show that the gene expression patterns in lymph node metastases are most similar to the corresponding primary tumors from which they arose, as long as samples contain sufficient proportions of tumor cells. Strikingly, gene expression patterns of metastatic primary HNSCC are largely maintained upon spread to the lymph node. Only a single gene, *metastasis-associated gene 1 (MTA1)*, was found to show consistently changed expression between a large number of matched primary tumor-lymph node metastasis pairs. The maintained expression pattern includes the predictive signature for HNSCC lymph node metastasis. These results underscore the importance of the primary tumor gene expression profile for development and treatment of metastasis. The findings also agree with the concept that disseminated cancer cells alter the surrounding tissue into a metastatic environment that resembles the primary tumor microenvironment. (Cancer Res 2006; 66(23): 11110-4)

Introduction

Metastasis is the process whereby primary tumor cells spread to distant sites in the body and is the leading cause of cancer-related deaths. In the conventional model, metastatic potential is acquired late during tumorigenesis as a result of sequential selective steps, with only small subpopulations of tumor cells gaining metastatic capacity and disseminating to other organs (1). Lately, this long-established model has been challenged by the results of gene expression profiling studies aimed at determining metastatic phenotypes (2-4). Use of complete tumor sections in such studies indicates that within primary tumors that have metastasized, most tumor cells exhibit a metastatic profile. This implies that metastatic potential has been acquired early and sustained throughout primary tumor development (5).

In line with this new model, metastases too may be similar to the primary tumor from which they originate. An important characteristic of such similarity is exhibition of a similar gene expression pattern. Maintenance of gene expression patterns during development of distant metastasis has indeed recently been shown for hematogenous spread of breast cancer (6). Such findings have important implications for our understanding of how metastasis develops and for cancer treatment. It is therefore imperative to determine whether maintenance of primary gene expression patterns during metastasis is a general property or whether this is restricted to certain types of cancer.

Recently, primary tumor gene expression signatures have been discovered that are predictive for the presence of lymph node metastasis for head and neck squamous cell carcinoma (HNSCC; refs. 3, 7, 8). In contrast to breast cancer, head and neck cancer spreads almost exclusively via a lymphatic route in which the cervical lymph nodes are the first location (9). One explanation for this is that in the lymphatic metastatic cascade exhibited by HNSCC, additional alterations take place in the lymph nodes that are required for cancer cells to spread further to distant sites via the blood (10).

Here, we have investigated whether HNSCC primary tumor gene expression patterns are preserved upon spread to lymph nodes. Strikingly, we find that gene expression profiles of primary metastatic tumors are maintained in their corresponding lymph node metastases. Besides playing a role during initiation of metastasis, the gene expression pattern present in the primary tumor, therefore, likely also plays an important role in survival and proliferation of metastatic tumor cells in lymph nodes. The results also shed light on development of distant metastases through the lymphatic route.

Materials and Methods

Detailed patient information and primary tumor selection criteria have been described previously (3). Samples taken from surgically removed cervical lymph node tissue were scored for the presence of metastases and, if positive, included for analysis of gene expression. Tissue scoring and sectioning, RNA isolation, mRNA amplification, cRNA labeling, hybridization, scanning, and preprocessing of expression data were done as previously described (3). Microarray layout, expression data, and protocols have been deposited in the public microarray database ArrayExpress with accession nos. A-UMCU-3 and E-TABM-114, respectively. Nineteen pairs of matched oral cavity/oropharynx primary tumor and lymph node metastases were expression profiled in dye-swap duplicate against a previously used reference pool of primary HNSCC samples (3). Following scanning, quantification, and data preprocessing, 2,135 genes were identified as differentially expressed in at least half of the analyzed samples ($P < 0.01$). The samples were hierarchically clustered based on their Euclidian distance measurement across the 2,135 differentially expressed genes. The "within-pair/between-pair scatter ratio" (WPBPSR; ref. 11) was determined by

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calculating the ratio of the within-pair similarity of a matched metastasis and primary tumor samples versus the average similarity of random samples (between-pair similarity). A low WPBPSR (<1.0) indicates a high similarity between the matched primary tumor and metastasis. The statistical significance of the similarity measurement was determined by a permutation test in which the WPBPSR was calculated for 10,000 random pairings (11).

Identification of changes in gene expression between the primary tumors and the lymph node metastases was done using SAM (<http://www-stat.stanford.edu/~tibs/SAM/>). With a false discovery rate of 5%, only one gene [*metastasis-associated gene 1 (MTA1)*, $q = 0$] was identified as differentially expressed between primary tumor and lymph node metastases. Metastatic signature outcome of the analyzed samples was determined as described previously (3, 12). Based on the expression pattern of the signature genes, a correlation was calculated with the average HNSCC metastatic profile. Positive correlation indicates a metastatic expression profile (N^+), and negative correlation indicates a non-metastatic profile (N_0). The previously published lists of genes within the 102 (3) and 825 (12) gene predictors used for the analyses in Fig. 3 are also available at <http://www.genomics.med.uu.nl/publications/pr/hnsc/>.

Results

Treatment of head and neck cancer patients with lymph node metastatic disease involves removal of the primary tumor and of cervical lymph node tissue that contains the metastatic lesions. To investigate the relationship between primary tumors and lymph node metastases, we first selected 14 pairs of primary HNSCCs and their matching cervical lymph node metastases

(Table 1). The primary tumors originated in the oral cavity or oropharynx and consisted of at least 50% tumor cells. The 14 corresponding lymph node metastases samples had a tumor percentage of at least 25%.

Gene expression profiles were generated using DNA microarrays that contained 70-mer oligonucleotides representing >21,000 genes (3). After normalization and statistical analyses, 2,135 genes were identified as differentially expressed in at least half of the samples. Unsupervised hierarchical clustering based on similarity measurements across the 2,135 differentially expressed genes grouped together 8 of the 14 primary tumor-lymph node metastasis pairs (Fig. 1A). For these eight clustered pairs, the metastasis sample was most similar to its matching primary tumor. For five of the six pairs that did not cluster together as a pair, the lymph node metastasis sample contained <50% tumor cells (Fig. 1A; Table 1), indicating that a lower tumor percentage within the lymph node samples was responsible for reduced pairing of samples. In agreement with this, lymph node samples that did not cluster pairwise with their corresponding primary tumor did themselves cluster together (Fig. 1A, right).

The idea that lower lymph node tumor percentage is responsible for reduced pairing of samples was further investigated by including five pairs for which the metastasis sample contained <10% tumor cells (Table 1), hereafter called "low tumor percentage pairs." The five additional lymph node metastasis samples clustered within the separate lymph node sample group and showed no direct clustering with their corresponding primary tumor samples (Fig. 1B).

Table 1. Patient and sample characteristics

Patient	Gender	Age	Primary tumor			Lymph node metastasis, tumor %				
			Location	Size (cm)	Tumor %		>75	50-75	25-50	<10
					>75	50-75				
1	M	55	Oral cavity	3.0	+			+		
2	F	62	Oral cavity	4.2	+		+			
3	M	67	Oropharynx	7.3			+			
4	M	52	Oral cavity	2.3	+			+		
5*	F	38	Oral cavity	4.0					+	
7	M	51	Oral cavity	3.0	+			+		
9*	M	61	Oropharynx	3.5	+				+	
10*	M	42	Oropharynx	5.5					+	
11*	F	55	Oral cavity	6.0	+				+	
13	M	82	Oral cavity	2.5	+		+			
14	F	52	Oropharynx	7.0	+		+			
17	M	61	Oral cavity	2.9	+			+		
18*	M	50	Oral cavity	3.0	+				+	
19*	M	66	Oral cavity	5.0	+				+	
6 [†]	M	50	Oral cavity	4.0					+	
8 [†]	M	58	Oropharynx	3.0					+	
12 [†]	F	38	Oropharynx	4.0	+				+	
15 [†]	F	63	Oropharynx	5.0					+	
16 [†]	M	46	Oral cavity	5.2	+				+	

NOTE: Primary tumors and lymph node metastasis samples are grouped according to their tumor percentage. Age is age at diagnosis; size is diameter of tumor in cm.

*Patients of whom the analyzed metastasis sample contained <50% tumor cells.

[†] Comprises five sample pairs for which the lymph node metastases samples contain <10% tumor cells and are referred to as low tumor percentage pairs.

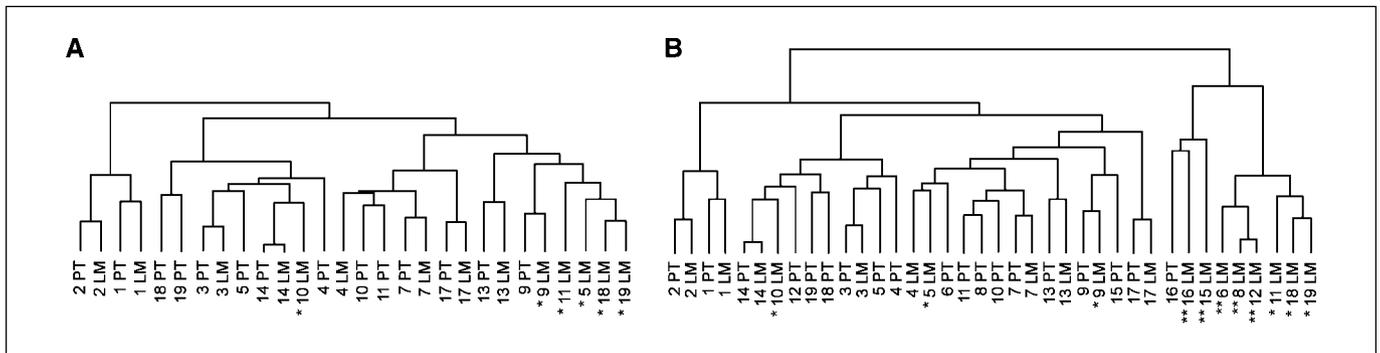


Figure 1. Hierarchical clustering of lymph node metastases (LM) and the primary HNSCC tumors from which they originated (PT). A, clustering of 14 sample pairs of which the metastasis samples contained at least 25% tumor cells. B, including five pairs of which the lymph node metastasis sample contained no or very little tumor cells (as in A). *, patients of whom the analyzed metastasis sample contained <50% tumor cells. **, comprises five sample pairs for which the lymph node metastases samples contain <10% tumor cells and are referred to as low tumor percentage pairs.

In summary, eight of the nine sample pairs that contained >50% tumor cells co-clustered as a pair. This analysis shows that gene expression profiles of lymph node metastases are most similar to the primary tumor from which they originate, provided that both samples contain enough tumor cells.

The significance of similarities between matched lymph node metastases and primary tumors was further analyzed by calculating a WPBPSR (11). A low WPBPSR (<1.0) represents a high similarity between matched samples. Thirteen of the 15 pairs with a metastasis sample that contained at least 25% tumor cells had a WPBPSR <1, indicating that these paired samples were more similar to each other than to the other samples (Fig. 2A). The higher WPBPSR for pairs with lower tumor percentage in the metastasis sample shows the importance of tumor cell percentage for the similarity in expression profiles (Fig. 2B). The average WPBPSR for matched metastases and primary tumors was 0.74 ($P < 0.0001$) when excluding, or 0.83 ($P = 0.004$) when including, the low tumor percentage pairs (Fig. 2C), showing that the observed WPBPSRs were not the result of random chance. Instead, the lymph node metastases expression patterns are more similar to the primary tumor from which they originated.

Although the overall gene expression patterns of lymph node metastases are similar to that of their corresponding primary tumors, this does not exclude the possibility of a small subset of genes with systematically changed expression upon spread to the lymph nodes. We therefore further investigated the similarity in gene expression upon lymph node metastasis by searching for genes that exhibited consistent changes in expression between a large number of primary tumors and their corresponding lymph node metastases. At a false discovery rate of 5%, only one differentially expressed gene could be identified. *MTAI* shows reduced expression in all 13 metastasis samples compared with the matched primary tumors with a statistically significant down-regulation ($P < 0.05$) in six samples. *MTAI* is a component of the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex and has previously been shown to be a regulator of metastatic potential by controlling the epithelial-to-mesenchymal transition (13). Increased expression of *MTAI* is associated with progression to the metastatic state in primary tumors (14). Here, we find reduced expression of this gene in the lymph node metastases compared with the primary tumors, suggesting that *MTAI* is only needed at the site of primary tumor. That only one gene is found to

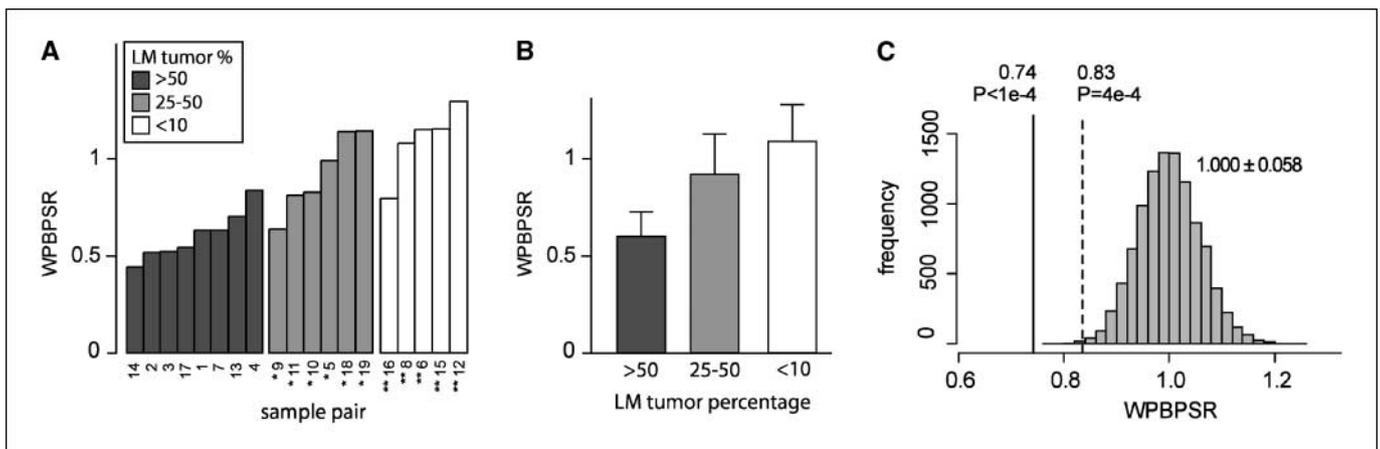


Figure 2. WPBPSR of matched individual pairs of primary tumors and lymph node metastases (A) grouped according to the tumor cell percentage of the lymph node metastasis samples (B). Pairs with a metastasis sample containing >50%, 25% to 50%, or <10% tumor cells are colored dark gray, light gray, and white, respectively. *, patients of whom the analyzed metastasis sample contained <50% tumor cells. **, comprises five sample pairs for which the lymph node metastases samples contain <10% tumor cells and are referred to as low tumor percentage pairs. Bars, SE of WPBPSR. LM, lymph node metastasis sample. C, Permutation test of the overall WPBPSR outcome. The histogram displays the WPBPSR distribution for 10,000 random pairings of the studied samples (1.000 ± 0.058). Solid line, WPBPSR of the 14 samples with at least 25% tumor cells in the metastasis sample (0.74; $P < 0.0001$). Dashed line, WPBPSR of all 19 pairs, including the five low tumor percentage pairs (0.83; $P = 0.0004$).

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be differentially expressed by this as well as other statistical analyses suggests that across the samples analyzed here, no specific pathway is consistently affected during subsequent development of lymph node metastasis after detachment and spread of cancer cells from the primary HNSCC tumor.

Preservation of general gene expression patterns upon spread of HNSCC primary tumors suggests that the previously identified metastatic signature (3) is also maintained upon metastasis to the lymph node. Analysis of expression of the 102 signature genes reveals that the metastatic signature profile within lymph node metastases is highly similar to the profile of the corresponding primary tumors (Fig. 3A). Consequently, the metastatic signature outcome based on the 102-gene profile was almost identical for the matched primary tumor and lymph node metastasis samples ($P < 0.01$) and opposite to the outcome for non-metastatic (N_0) primary tumors (Fig. 3B). Metastatic signature outcome based on the gene profile of an extended set of 825 signature genes (12) also showed a similar outcome for the lymph node metastases compared with the primary tumors ($P < 0.01$; Fig. 3C). In one case, the metastasis sample showed a stronger metastatic profile than its primary tumor (Fig. 3B and C, no. 4). And in one case, a reduction in metastatic profile for the lymph node sample was observed, which could be explained by the reduced tumor percentage in this sample (no. 10). As is discussed below, strong preservation of the primary tumor metastatic gene profile during metastases development (Fig. 3C) underlines the importance of the identified signature genes, not only for their association with metastatic potential at the site of the primary tumor but also for further development and treatment of lymph node metastases.

Discussion

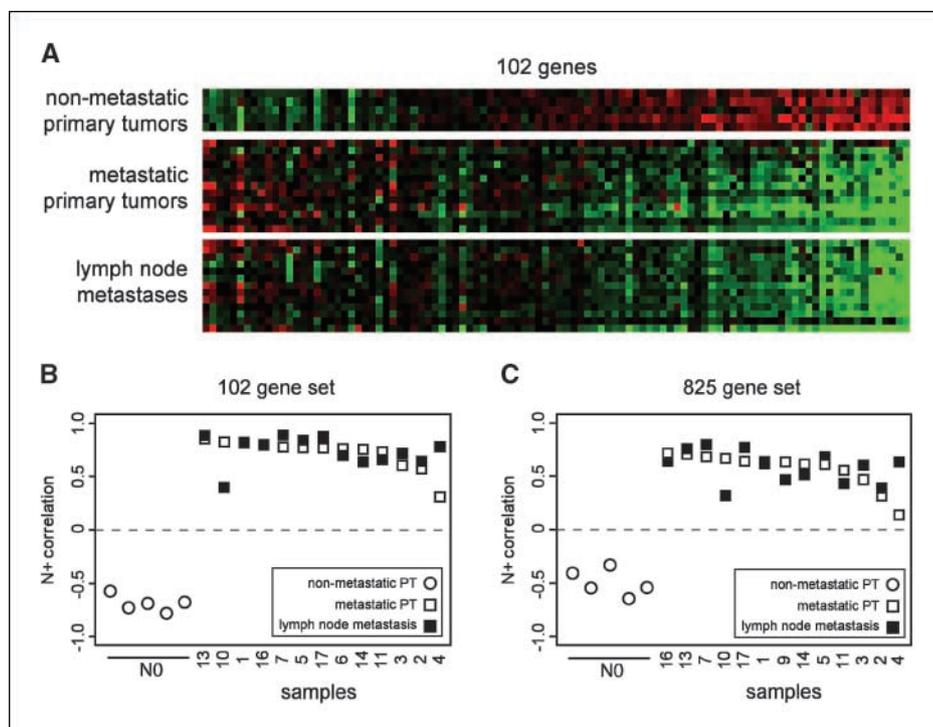
Recently, metastatic signatures for primary oral cavity and oropharynx squamous cell carcinomas have been identified that

are capable of identifying which tumors have metastasized to the cervical lymph nodes (3, 7, 8). Here, we show that lymph node metastases reflect both the general and the metastatic gene expression patterns of primary oral cavity and oropharynx tumors from which they originate. Apparently, the different environment does not effect the overall gene expression to such a degree that the metastases are markedly different from the primary tumor. As the expression profile contains both tumorous as well as stromal genes, this finding indicates that the disseminated cancer cells alter their adjacent stroma into a "metastatic" microenvironment that is similar to the primary tumor microenvironment in which they can survive and proliferate (15).

For breast cancer, it has previously been shown that primary tumor gene expression patterns are maintained upon metastasis (11). For development of HNSCC lymph node metastasis, we show here that the primary tumor gene expression patterns are also maintained. These findings indicate that disseminated primary tumor cells do not need to undergo additional developmental changes to survive and proliferate in metastatic sites and supports the theory that metastatic properties are acquired early during tumorigenesis and sustained through cancer progression (5). In addition, the observed stability in gene expression patterns are in line with the proposal that metastasis gene expression signatures are based on genetic background and influenced too a lesser degree by specific tumorigenic processes (16).

Primary breast tumor cells are directly suitable for distant spread via the hematogenous route without involvement of regional lymph nodes (10). Head and neck cancer, however, nearly always spreads to distant sites via the lymph nodes as an intermediate stage before further hematogenous spread (9). It has therefore previously been postulated that distant dissemination of head and neck cancer depends on the presence of lymph node metastasis, presumably due to additional developmental changes in the solid lymph node metastases that are required for hematogenous distant

Figure 3. Metastatic signature profiles for lymph node metastases and primary tumors. **A**, expression profiles of the 102 predictive gene set for non-metastatic and metastatic primary tumors and for lymph node metastases. *Red*, up-regulation; *green*, down-regulation. **B** and **C**, signature correlative outcome of 14 metastatic primary tumors (*open squares*) and 14 matched lymph node metastases (*closed squares*) together with five previously analyzed non-metastatic primary tumors (*open circles*). Signature outcome (N^+ correlation) was based on (**B**) the previously identified 102 predictive genes (3) or (**C**) on the extended set of 825 metastatic predictive genes (12). Positive correlation indicates a metastatic gene expression profile (N^+) and negative correlation a non-metastatic profile (N_0).



spread (9, 10). It is therefore striking that the lymph node metastases samples analyzed here show no obvious alterations in gene expression compared with their primary tumors. Apparently, the local lymph nodes provide a fertile environment for development of metastasis. Our results indicate that once the primary tumor has gained the metastatic phenotype, few further alterations in gene expression within the metastatic tumor cells are required for tumor establishment in the lymph nodes.

These findings underscore the importance of understanding the roles of genes found to be associated with metastasis through expression profiling studies. Within the large set of 825 genes found by multiple sampling (12), several significant groups of shared function can be found as well as numerous putative regulatory links. For example, several cytokines and immune regulatory genes are represented, which is in agreement with studies linking malignant progression and high risk with activation of nuclear factor- κ B signaling (17, 18). More exhaustive analyses of metastasis associated gene expression will determine the presence of other common features, identifying the most promising targets for therapy.

Although this study indicates that no additional developmental changes take place upon spread of primary HNSCC tumors to the regional lymph nodes, we cannot completely rule out the possibility that extra alterations are needed for subsequent spread of cancer cells from the established lymph node metastases to distant sites via the blood. Alterations in a small subpopulation of tumor cells within the solid lymph node metastasis could lead to subsequent hematogenous spread and survival at distant sites (1). Alternatively, these changes might not occur in the solid lymph node metastases but afterwards when individual cancer cell circulate through the lymph fluid before entering the blood vessels (19). To be certain whether additional alterations are needed for subsequent distant spread

of HNSCC, distant metastases need to be analyzed and compared with corresponding primary tumor and lymph node metastases. However, from the head and neck cancer patients studied here, no distant metastases were available for gene expression analysis.

The maintenance of general primary tumor gene expression patterns upon dissemination of breast cancer (11) and of HNSCC, as shown in this study, indicates that metastatic primary tumor cells already harbor properties for survival and proliferation in foreign sites. Finding two such cases of primary tumor expression profile maintenance makes it more likely that it is a general property of cancer progression. The previously identified HNSCC metastatic gene expression profile is present in the primary tumor during initiation of metastasis and within developing lymph node metastases, indicating a role during initiation of metastasis and for survival and proliferation in the lymph nodes. Because this signature is present only in metastasizing primary head and neck tumors (3) and is retained in the lymph node metastases (this study), therapeutic targeting of its components forms a rational approach towards preventing or impeding lymph node metastasis, especially when targeting the stable metastatic microenvironment that enables cancer cells to survive and proliferate at remote sites (15, 20).

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