High-Throughput Copy Number Analysis of 17q23 in 3520 Tissue Specimens by Fluorescence *in Situ* Hybridization to Tissue Microarrays

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The chromosomal region 17q23 has been shown to be commonly amplified in breast tumors, especially those with poor prognosis. In addition to breast cancer, studies by comparative genomic hybridization have implicated the involvement of 17q23 in other tumor types as well. Here we performed a large-scale survey on the distribution and frequency of the 17q23 copy number increases across different tumor types using fluorescence *in situ* hybridization on tissue microarrays containing 4788 specimens. A total of 4429 tumor samples representing 166 different tumor categories and 359 normal tissue samples from 40 different tissue categories were analyzed. Successful hybridizations were observed in 3520 of the 4788 specimens (74%). Increased 17q23 copy number was detected in 15% of the evaluable specimens with tumors originating from the lung, mammary gland, and soft tissue being most frequently affected. Interestingly, high-level amplification was detected only in 2% of the tumors and was generally restricted to mammary tumors. In addition, we observed an association between the frequency of increased 17q23 copy number and tumor progression in various tumor types. These results indicate that increased 17q23 copy number occurs frequently in several different tumor types suggesting that increased dosage of genes in this region might play a role in development and progression of many tumor types. *(Am J Pathol 2002, 161:73–79)*

Development and progression of cancer is driven by a step-wise accumulation of genetic alterations. Activation of oncogenes and other growth-promoting genes is an important part of this process and gene amplification is one of the mechanisms that leads to the activation of such genes. Several different chromosomal regions have been shown to be frequently amplified in human tumors and, in some instances, subsequent studies have successfully identified genes from these amplified regions that have a critical role in cancer development.[1,2]

Genome-wide copy number analysis by comparative genomic hybridization has illustrated that 17q23 is one of the most frequently amplified chromosomal regions in breast cancer.[3,4] Recently, the 17q23 amplification was shown to be associated with poor prognosis of breast cancer[5] indicating that an increased dosage of one or more genes in this region is involved in the progression of this disease. In addition to breast cancer, increased copy number of the 17q23 region has also been observed by comparative genomic hybridization in tumors of the adrenal gland,[6] brain,[7] esophagus,[8] lung,[9,10] ovary,[11] stomach,[12] urinary bladder,[13] and uterus[14–16] indicating that 17q23-specific genes may also be important in the development of other tumor types.

Although the 17q23 amplification has been observed by comparative genomic hybridization in several different tumor types, these analyses have not covered the entire tumor spectrum and usually only a limited number of samples from a given tumor type has been analyzed. In addition, detailed analysis of the 17q23 amplicon has only been performed in breast cancer where we recently performed a microarray-based characterization of the 17q23 amplicon in breast cancer cell lines and in a large collection of primary breast tumors.[17] This analysis illustrated that in most breast cancers the amplification extends over a very large region at 17q23 and leads to simultaneous activation of several genes.[17]

The purpose of the present study was to survey the distribution and frequency of the 17q23 amplification across a large variety of tumor types. We used the recently developed tissue microarray (TMA) technology in which hundreds of individual tumor biopsies from archival paraffin-embedded tissue blocks are arrayed into a new paraffin block.[18] Sections from such a TMA can be used for parallel high-throughput analysis of hundreds of tumor samples in a single experiment. Here, we studied the 17q23 amplification *in vivo* in 3520 tissue specimens using fluorescence *in situ* hybridization (FISH) to TMAs.

Materials and Methods

TMA

A total of 4788 tissue samples, representing 3709 primary tumors (from 135 different tumor categories), 720 metastases (of 31 different tumor categories), and 359 normal tissues (of 40 different tissue categories), were used in this study. The tissue samples were derived from the following organs or anatomical sites: fetus and placenta, brain, salivary glands, oral cavity, esophagus, stomach, small intestine, colon, appendix, anus, gallbladder, pancreas, liver, larynx, lung, kidney, urinary bladder, prostate, testis, ovary, uterus, vagina and vulva, mammary gland, adrenal gland, thyroid gland, parathyroid gland, thymus, nodal and lymphatic tissue, skin, and soft tissue.

The tissue specimens were placed on 10 different TMA blocks, each containing ~500 specimens. The TMAs were constructed as previously described.[18] Briefly, a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD) was used to create holes in a recipient paraffin block and to acquire cylindrical core tissue biopsies with a diameter of 0.6 mm from histologically representative areas of the donor blocks. The tissue core biopsies were transferred to the recipient paraffin block at defined array positions. Each tumor was represented only once on the array. Five-μm sections were cut from the TMA block using a microtome and an adhesive-coated tape-sectioning system (Instrumedics, Inc., Hackensack, NJ).

Copy Number Analysis by FISH

Two overlapping bacterial artificial chromosome clones (hRPK.15_K_2/AC005901 and hRPK.332_H_18/AC005746) were selected for FISH analysis. These clones represent the previously identified core region of the 17q23 amplification in breast cancer[17] and include the TBX2 gene that has been previously implicated as one of the putative targets for the 17q23 amplification in breast cancer.[19–21] The clones were labeled with SpectrumOrange (Vysis Inc., Downers Grove, IL) using random priming. A SpectrumGreen-labeled chromosome 17 centromere probe (Vysis) was used as reference.

Dual-color FISH was performed as previously described.[22] In brief, TMA sections were treated according to the Paraffin Pretreatment Reagent kit protocol (Vysis), denatured at 94°C for 5 minutes in Tth-buffer [10
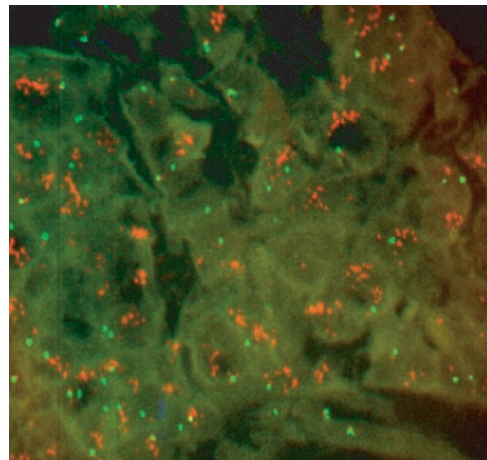
Figure 1. FISH analysis on TMA. The SpectrumOrange-labeled 17q23-specific probe (red signals) and SpectrumGreen-labeled chromosome 17 centromere probe (green signals) were hybridized to the TMAs. An example of a ductal carcinoma of the breast tumor sample with high-level amplification of 17q23 is shown.

mmol/L Tris-HCl, pH 8.9 (25°C), 0.1 mol/L KCl, 1.5 mmol/L MgCl, 50 μg/ml bovine serum albumin, 0.05% Tween 20 (v/v)], treated with Proteinase K (10 μg/ml in phosphate-buffered saline) at 37°C for 10 minutes, dehydrated, and air-dried. Hybridization was performed overnight. After hybridization the slides were washed in 0.4× standard saline citrate/0.3% Nonidet P-40 at 72°C for 3 minutes and then counterstained with 4′, 6-diamidino-2-phenylindole in anti-fade solution.

FISH signals were evaluated using a Zeiss fluorescence microscope. Specimens were classified into three groups (normal, gain, and high-level amplification) based on the ratio between the 17q23 and the 17 centromere signals in the tumor cells. A minimum of 50 tumor cells was evaluated per specimen. The samples with a ratio of 1:1 were considered normal. If more than 10% of the tumor cells had a ratio different from 1:1 the specimens were classified as either gain (1:1<ratio <3:1) or high-level amplification (ratio ≥3:1 or tight clusters of signals).

Statistical Analysis

The association between increased 17q23 copy number and tumor progression was analyzed using Fisher's exact test. All *P* values are two-sided.

Results

17q23 Copy Number Analysis by FISH to TMAs

The 17q23 copy number was determined *in situ* in 4788 tissue specimens. The tissue samples included 3709 primary tumors (from 135 different tumor categories), 720 metastases (from 31 different tumor categories), and 359 normal tissues (from 40 different tissue categories). Successful hybridizations were observed in 3520 of the 4788 specimens (74%) in the TMAs (Figure 1). The failures were because of missing or unrepresentative samples (9%), autofluorescence of the tissue (10%), or poor hy-

bridization quality (7%). Tissues containing large amounts of fat, such as lipomas, were most frequently nonanalyzable, most likely because of the melting of the fat during the hybridization procedure.[22]

Analysis of the normal tissue specimens demonstrated that all except 1 of the 245 evaluable samples showed the expected two signals for both 17q23 and centromere 17 probes. A single specimen of normal liver showed five signals for 17q23 and four signals for 17 centromere. A retrospective histological analysis using an adjacent section from the TMA did not reveal any abnormal cells in this particular sample. As normal liver cells can be polyploid[23] we expect that this particular sample represents such a case.

Frequent Increase in 17q23 Copy Number in Several Tumor Types

Evaluable hybridization results were obtained from a total of 3275 tumor samples and increased copy number of the 17q23 region was detected in 477 (15%) samples representing 86 (52%) of the 166 tumor categories examined (Table 1). The frequency of increased copy number varied considerably from one tumor type to another with 65 tumor categories having at least 10% of the specimens affected (Table 1). Several organs or anatomical sites, such as brain, lung, mammary gland, prostate, skin, soft tissue, and thyroid gland, showed increased copy number in multiple tumor categories (Table 1). Increased copy number was particularly frequent in the lung, mammary gland, and soft tissue tumor categories (Figure 2).

For example, a total of 45 (35%) of the 129 interpretable lung tumor specimens showed increased copy number with large cell carcinomas (56% of the cases) and metastases of lung adenocarcinomas (50%) being most frequently affected. In addition, increased 17q23 copy number was also observed in adenocarcinomas (32%), squamous cell carcinomas (30%), small cell carcinomas (20%), squamous cell carcinoma metastases (17%), and carcinoid tumors (9%) of the lung (Table 1, Figure 2). Similarly for tumors of the soft tissue, 76 (21%) of the 355 analyzable samples showed increased copy number with tumor categories, such as neuroepithelioma (PNET), rhabdomyosarcoma, malignant fibrous histiocytoma, and malignant mesothelioma, demonstrating increased copy number in more than half of the samples (Table 1, Figure 2). Other very frequently affected tumor categories included oligodendroglioma, hormone refractory prostate cancer, squamous cell carcinoma of the larynx, leiomyosarcoma, and metastasis of ductal invasive breast cancer (Table 1). Increased copy number of 17q23 was also observed in several tumor types that were represented by a small number of specimens (less than 10 analyzable samples) in the TMAs (Table 1). For example, gain of 17q23 was detected in a single sample of angiosarcoma, three of four medulloblastomas, and in two of three metastases of pancreatic adenocarcinoma.

As expected, increased copy number of 17q23 was frequently observed in the tumor categories originating from the mammary gland. Out of the 589 mammary tumor samples analyzed 176 (30%) showed increased 17q23 copy number (Table 1). The frequency of increased copy number varied among the different subtypes of mammary tumors. The most frequently affected tumor categories were metastases of ductal invasive carcinomas (40% of the cases), mucinous carcinomas (27%), and ductal carcinomas (22%).

High-Level Amplification of 17q23

Increased copy number in the form of high-level amplification of 17q23 was observed in 65 specimens (2%) distributed throughout 19 tumor categories representing adrenal gland, lung, mammary gland, ovary, skin, soft tissue, stomach, thyroid gland, urinary bladder, and uterus (Table 1). Even though high-level amplification was observed in tumors from several organs the majority of affected specimens (78%) were derived from the mammary gland (Table 1, Figure 2). The frequency of high-level amplification varied from 1 to 15% among the different malignancies. Invasive ductal breast cancer was the most common cancer subtype exhibiting 17q23 amplification (Table 1). Interestingly, there was also evidence for frequent high-level amplification in some rare tumor types of which only a few samples could be analyzed, such as anaplastic thyroid carcinomas (amplification in two of the six samples analyzed), metastases of adenocarcinoma of papilla vateri (two of two), and dermatofibrosarcoma (one of four).

Analysis of Primary Tumors and Metastases

Increased copy number of 17q23 was observed in an average of 11% of primary tumors and 33% of metastases. For example, the average frequency of increased copy number was 14% in primary mammary tumors and 36% in breast cancer metastases. Similarly, increased 17q23 copy number was rare in untreated primary prostate tumors (3%) whereas it was frequent in late-stage prostate tumors (46% of hormone refractory adenocarcinomas and 31% of metastases). Furthermore, only 5% of the noninvasive transitional cell carcinomas of the urinary bladder were affected whereas 17% of the invasive tumors showed increased copy number. Statistical analysis in tumor categories in which both primary tumors and metastases were available showed a clear association between increased copy number at 17q23 and tumor progression (Table 2). This association was statistically significant for colon adenocarcinomas, mammary ductal carcinomas, and prostate adenocarcinomas (Table 2) as well as when all primaries and metastases were combined ($P < 0.0001$).

Discussion

This study represents the first large-scale analysis of copy number changes in thousands of tissue samples across all different tumor types using FISH to TMAs and

Table 1.   Increased 17q23 Copy Number by FISH Analysis on TMA

| Organ/anatomic site	Tumor category	No. of tumors*	Increased copy number (%)	High-level amplification (%)
Adrenal gland	Pheochromocytoma	20	30	5
Brain	Astrocytoma	22	9	0
Brain	Glioblastoma multiforme	31	6	0
Brain	Meningeoma	28	14	0
Brain	Oligodendroglioma	15	47	0
Colon	Adenocarcinoma	276	0.4	0
Colon	Adenocarcinoma (metastases)	32	9	0
Esophagus	Squamous cell carcinoma	15	33	0
Gallbladder	Adenoma	13	23	0
Hematology	Non-Hodgkin lymphoma	84	6	0
Larynx	Squamous cell carcinoma	21	43	0
Liver	Hepatocellular carcinoma	47	17	0
Lung	Adenocarcinoma	28	32	4
Lung	Carcinoid	11	9	0
Lung	Large cell carcinoma	34	56	0
Lung	Small cell carcinoma	15	20	0
Lung	Squamous cell carcinoma	27	30	0
Mammary gland	DCIS	21	10	5
Mammary gland	Invasive carcinoma, ductal	41	22	15
Mammary gland	Invasive carcinoma, ductal (metastases)	342	40	11
Mammary gland	Invasive carcinoma, lobular	28	7	0
Mammary gland	Invasive carcinoma, lobular (metastases)	69	16	4
Mammary gland	Invasive carcinoma, medullary	32	13	0
Mammary gland	Invasive carcinoma, mucinous	11	27	9
Metastases	Adenocarcinoma (unknown primary)	16	44	0
Oral cavity	Squamous cell carcinoma	19	5	0
Oral cavity	Squamous cell carcinoma (metastases)	26	12	0
Ovary	Adenocarcinoma, endometroid	35	11	3
Ovary	Adenocarcinoma, serous	45	9	0
Pancreas	Adenocarcinoma	22	14	0
Parathyroid	Adenoma	17	18	0
Prostate	Adenocarcinoma (metastases)	13	31	0
Prostate	Adenocarcinoma, hormone refractory	13	46	0
Prostate	Adenocarcinoma, untreated	63	3	0
Salivary glands	Adenolymphoma	25	4	0
Skin	Cylindroma	17	12	0
Skin	Malignant melanoma	63	16	0
Skin	Squamous cell carcinoma	28	14	4
Soft tissue	Leiomyosarcoma	46	41	0
Soft tissue	Liposarcoma	22	32	0
Soft tissue	Malignant fibrous histiocytoma	18	50	0
Soft tissue	Malignant mesenchymoma	10	10	0
Soft tissue	Malignant mesothelioma	10	50	0
Soft tissue	Malignant schwannoma	10	10	10
Soft tissue	Neurofibroma	23	9	0
Soft tissue	PNET	19	58	0
Soft tissue	Rhabdomyosarcoma	14	57	0
Soft tissue	Schwannoma	45	11	0
Stomach	Adenocarcinoma	113	15	2
Thymus	Thymoma	20	5	0
Thyroid gland	Adenoma	23	13	0
Thyroid gland	Carcinoma, follicular	29	3	0
Thyroid gland	Carcinoma, medullary	13	8	0
Thyroid gland	Carcinoma, papillary	26	4	0
Urinary bladder	TCC, invasive	46	17	0
Urinary bladder	TCC, non invasive	74	5	1
Uterus	Adenocarcinoma, endometroid	117	4	0.9
Uterus, cervix	Carcinoma in situ	13	8	0
Uterus, cervix	Squamous cell carcinoma	24	4	0
Vagina and vulva	Squamous cell carcinoma, vulva	42	14	0
Brain	Medulloblastoma	4	75	0
Esophagus	Adenocarcinoma	3	33	0
Esophagus	Squamous cell carcinoma (metastases)	2	50	0
Larynx	Squamous cell carcinoma (metastases)	3	33	0
Lung	Adenocarcinoma (metastases)	8	50	0
Lung	Squamous cell carcinoma (metastases)	6	17	0
Mammary gland	Adenocarcinoma (metastases)	7	43	0
Mammary gland	Invasive carcinoma, cribriform	6	17	17

Table 1. *Continued*

Organ/anatomic site	Tumor category	No. of tumors*	Increased copy number (%)	High-level amplification (%)
Mammary gland	Invasive carcinoma, medullary (metastases)	1	100	100
Mammary gland	Invasive carcinoma, mucinous (metastases)	1	100	100
Mammary gland	Invasive carcinoma, papillary	7	43	0
Metastases	Squamous cell carcinoma (unknown primary)	7	14	0
Oral Cavity	Adenocarcinoma (metastases)	2	50	0
Ovary	Adenocarcinoma (metastases)	5	20	0
Ovary	Carcinoma	2	50	0
Pancreas	Adenocarcinoma (metastases)	3	67	0
Papilla vateri	Adenocarcinoma (metastases)	2	100	100
Skin	Squamous cell carcinoma (metastases)	3	33	0
Soft tissue	Angiosarcoma	1	100	0
Soft tissue	Dermatofibrosarcoma	4	50	25
Soft tissue	Fibrosarcoma	6	50	0
Soft tissue	Haemangiopericytoma	5	40	0
Stomach	Adenocarcinoma (metastases)	7	29	0
Thyroid gland	Carcinoma, anaplastic	6	33	33
Uterus	Malignant muellerian mixed tumor	4	50	0
Uterus, cervix	Adenocarcinoma	4	25	0

*Number of interpretable tumors is shown. Tumor categories with less than 10 analyzable samples are shown at the bottom of the table.

thus illustrates the utility of TMA technology for large-scale surveys to characterize the disease specificity of genomic alterations at the population level. Here we used TMAs to evaluate the frequency of increased copy number at 17q23 in 4788 archival tissue specimens representing 206 different tissue categories. FISH on archival tissue sections can be difficult, and it is likely that for optimal performance case-by-case adjustments to hybridization protocols are required. However in this study, our overall success rate for FISH on TMAs constructed from archival specimens was 74% across a wide variety of tumor types. This result demonstrates that the concept of using FISH on multitumor TMAs is robust enough to be used as a screening tool for finding disease associations for molecular alterations.

The present study showed increased 17q23 copy number in 15% of the tumors representing 86 of the 166 different tumor categories analyzed. Increased copy number was observed frequently (in at least 10% of the specimens) in 65 of these categories, although there was considerable variation in frequency from one tumor type to another. The 166 different tumor categories evaluated
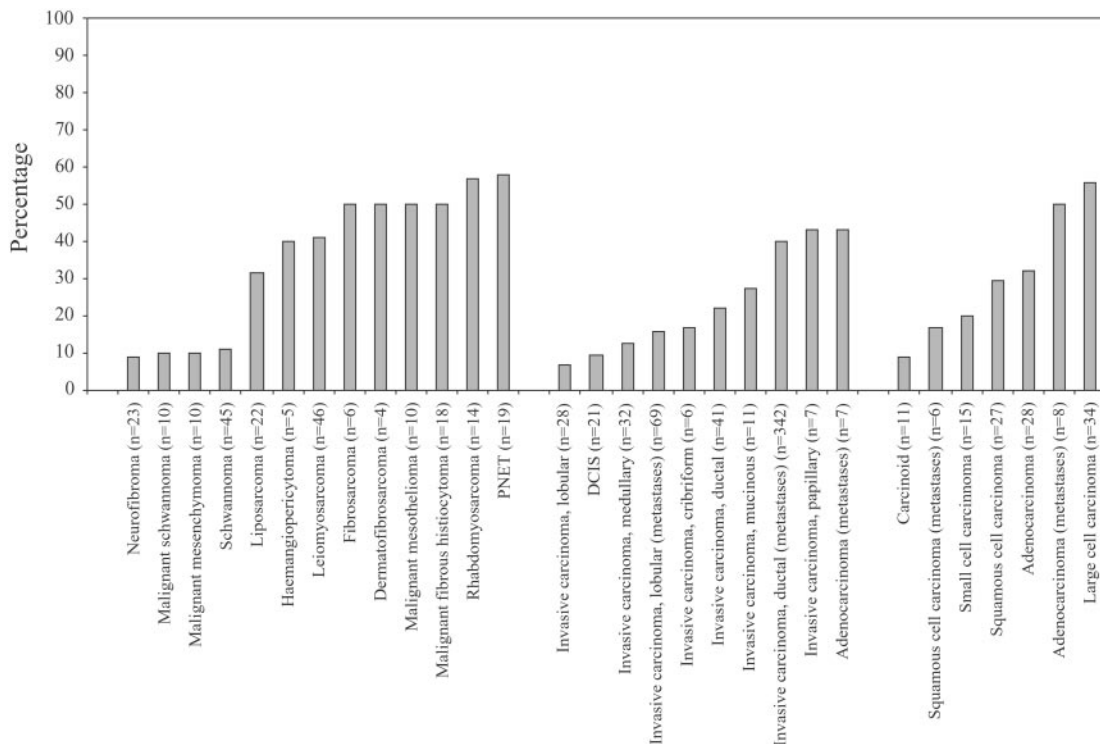
Figure 2. The frequency of increased 17q23 copy number in different tumor categories originating from lung, mammary gland, and soft tissue.

Table 2. Comparison on the Frequency of Increased 17q23 Copy Number Between Primary Tumors and Metastases from the Same Tumor Category*

Organ/anatomic site	Tumor category	Frequency of increased copy number (n[†])		Statistical significance
		Primary tumors	Metastases	
Colon	Adenocarcinoma	0.4% (276)	9% (32)	*P* = 0.004
Mammary gland	Carcinoma, ductal	22% (41)	40% (342)	*P* = 0.03
Mammary gland	Carcinoma, lobular	7% (28)	16% (69)	n.s.[‡]
Oral cavity	Squamous cell carcinoma	5% (19)	12% (26)	n.s.[‡]
Prostate	Adenocarcinoma	3% (63)	31% (13)	*P* = 0.007

*For tumor categories with 10 or more evaluable specimens.
[†]Total number of tumors evaluated.
[‡]Not significant.

in this study cover malignancies arising from almost every organ of the body. Interestingly, cancers emerging from only a few of these organs, such as small intestine, kidney, and testis, never exhibited abnormal 17q23 copy number. Based on our results, 17q23 copy number increases are common in human malignancies and may contribute to the development and progression of cancer in a variety of cellular environments.

The increased 17q23 copy number was observed most commonly in tumors derived from brain, lung, mammary gland, ovary, soft tissue, and urinary bladder. These results are in concordance with previous data from comparative genomic hybridization studies showing recurrent 17q23 gains in these tumor types.[3,4,7,9–11,13] We also observed increased 17q23 copy number in hormone refractory prostate cancer and prostate cancer metastases confirming a recent report on 17q23-q24 amplification in advanced prostate cancer.[24]

As expected, increased 17q23 copy number was most common in the mammary tumors. The frequency of increased copy number varied among different subtypes of mammary tumors with an average of 14% in primary tumors. These results confirm the previous data on the high frequency of the 17q23 amplification in primary breast cancer.[5,17,20,21] The data also show that increased 17q23 copy number is not restricted to certain histological subtypes of breast cancer. The only breast tumor subtypes in which 17q23 copy number increases were not observed were phylloides tumor, tubular and apocrine carcinomas, as well as metastases of papillary adenocarcinoma. However, only a relatively small number of samples representing these tumor categories were examined in this study.

High-level amplification of 17q23 was observed in only 2% of the tumor samples. Although these tumors belong to 19 different tumor categories, most of them originate from the mammary gland indicating that high-level amplification of 17q23 is rather specific to mammary tumors. Several other chromosomal regions that are frequently amplified in mammary tumors do not show such tissue specificity. For example, the 20q13 amplification that was initially identified in breast cancer[25] is commonly observed in several other tumor types, such as bladder, colorectal, gastric, and ovarian cancers.[26–29] These results suggest that mammary tumors might gain a special advantage of a very high 17q23 gene dosage, or that the

genes affected by this amplification have a special function in mammary tumorigenesis. It has to be noted, however, that this FISH study was performed using a custom-made 17q23-specific probe set that corresponds to the previously identified core region of the 17q23 amplification in breast cancer.[17] Although our previous data indicates that in breast tumors the region affected by increased copy number at 17q23 is rather large,[17] it is possible that smaller amplicons exist in other tumor types and that the location of the core of the amplicon might be different. Therefore, these amplicons could have been missed by the probe set used in this study.

Comparison of the frequency of increased 17q23 copy number between unmatched primary tumors and metastases illustrated a clear association between increased copy number and advanced tumor stage. This association was observed for all tumor categories in which primaries and metastases were available for analysis. In addition, a marked increase in the frequency of the 17q23 alterations was observed in transition from noninvasive to invasive bladder tumors and from untreated to hormone refractory prostate cancer. Together with the previously reported association between 17q23 amplification and poor prognosis in breast cancer,[5] these data strongly argue for an important role of one or several 17q23 genes for tumor progression.

Traditionally, discoveries of new amplification regions or putative amplification target genes originate from the analysis of a single tumor type. Subsequent studies are then required to evaluate the possible involvement of these newly discovered genes or loci in other tumor types. This is usually a laborious and slow process and therefore, leads to a considerable delay from the discovery of a potentially important genetic alteration to the definition of the tumor types in which it may play a role. In a pilot study using a mini multitumor TMA containing samples from 17 different tumor types, we have previously shown that representative frequencies of gene amplification can be obtained on TMAs.[30] Here we illustrate that using the TMA technology it is now possible to define virtually all tumor types that are influenced by a specific genetic alteration in one single high-throughput study of thousands of tumor specimens representing hundreds of different tumor types. The present study is the first in which almost all tumor types have been surveyed for the presence of a specific gene amplification. We predict

that the use of the TMA technology for such surveys will facilitate rapid translation of gene discoveries to therapeutic and diagnostic applications in the future.

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