High WT1 Expression Is Associated with Very Poor Survival of Patients with Osteogenic Sarcoma Metastasis

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Abstract

Purpose: Although metastasis is the primary determinant of poor survival of patients with osteogenic sarcoma, some patients live much longer than others, indicating metastatic heterogeneity underlying survival outcome. The purpose of the investigation was to identify genes underlying survival outcome of patients with osteogenic sarcoma metastasis.

Experimental Design: We have used microarray to first compare mRNA expression between normal bone and osteogenic sarcoma specimens, identified genes overexpressed in osteogenic sarcoma, and compared expression of the selected gene between a poorly metastatic (SAOS) and two highly metastatic cell lines (LM8 and 143B). Finally, expression of the selected gene was assessed by immunostaining of osteogenic sarcoma samples with known survival outcome.

Results: Microarray analysis revealed 5.3-fold more expression of WT1 mRNA in osteogenic sarcoma compared with normal bone and >2-fold overexpression in 143B and LM8 cells compared with SAOS. Furthermore, WT1 mRNA was absent in normal bone (10 of 10) by reverse transcription-PCR but present in osteogenic sarcoma – derived cell lines (5 of 8). One hundred percent (42 of 42) of low-grade osteogenic sarcoma specimens expressed no WT1 as determined by immunostaining; however, 24% (12 of 49) of the high-grade specimens showed intense staining. Mean survival of patients with high-grade metastatic osteogenic sarcoma but low WT1 staining (27 of 37) was 96.5 ± 129.3 months, whereas mean survival of patients with high-grade metastatic osteogenic sarcoma having intense staining (10 of 37) was 18.3 ± 12.3 months (P > 0.0143). All splice variants of WT1 mRNA, including a hitherto unknown variant (lacking exons 4 and 5), were found to be expressed in osteogenic sarcoma.

Conclusion: WT1 seems to be associated with very poor survival of patients with osteogenic sarcoma metastasis.

Osteogenic sarcoma is the most common neoplasm involving the skeleton that predominantly affects the children and the young (1–3). High grade and metastasis are the most useful prognostic indicators of clinical outcome of the disease. Preoperative chemotherapy followed by surgery remains the method of treatment for patients with the disease (4–6). Such a treatment regimen often produces satisfactory results, but novel avenues must be explored to improve survival outcome, which remains suboptimal. In fact, 5-year survival for patients with metastatic osteogenic sarcoma remains only 20% even after the introduction of chemotherapy three decades ago (7–9). No reliable molecular marker exists corresponding to any stage of the disease. It is, however, imperative that such markers are urgently needed for improved management of patients with osteogenic sarcoma. A molecular marker that can predict poor prognosis not only has implications for current treatment regimen, it may also serve as a novel target for the development of therapy against the most aggressive form of osteogenic sarcoma.

Metastasis remains the primary cause of poor survival of patients with osteogenic sarcoma; however, survival of patients with metastatic osteogenic sarcoma has a wide window, ranging from 4 to 400 months in our cohort of 91 patients (details discussed in Results). These observations hint at the presence of “metastatic heterogeneity” underlying the wide spectrum of patient survival and also raise questions on the nature of the relationship between metastasis and patient survival. In fact, we speculate that specific genetic factor besides factors underlying metastasis contribute to patient survival, and that such genetic factors are most active in the highly aggressive form of osteogenic sarcoma as estimated by very poor survival. Conceptually, metastatic heterogeneity may have overlapping...
characteristics with intratumoral heterogeneity (see, e.g., refs. 10–12); however, the latter phenomenon does not seem to distinguish between metastatic and nonmetastatic tumors, whereas metastatic heterogeneity is considered to be a phenomenon of metastatic tumors only. Consequently, delineation of metastatic heterogeneity is crucial to improving treatment options for patients with osteogenic sarcoma.

Current treatment decision for patients with osteogenic sarcoma entirely depends upon the anatomic location and histologic evaluation of the biopsy specimen, such that a standardized chemotheraphy regimen is initiated upon diagnosis of the disease. Such a treatment strategy fails to include the possibility of a lack of response to chemotherapy because there is no biological (molecular) marker able to distinguish between good versus poor responders. Our aim is to identify prognostic molecular markers, which, at the time of diagnosis, can predict survival outcome of patients with metastatic osteogenic sarcoma. In this article, we provide evidence for the WT1 gene to be such a marker, high expression of which seems to correlate with very poor survival of patients with osteogenic sarcoma metastasis.

Materials and Methods

Patient specimens. The osteogenic sarcoma biopsy specimens were obtained from investigators within and outside of the Institution. In all cases, Institutional Review Board–approved protocols were followed to collect specimens. The 10 osteogenic sarcoma specimens used for microarray analysis were all collected in the past 5 years: five of which were diagnosed as grade 4 (highest grade; of these, two patients died of the disease already, and one is alive with metastasis); samples from three patients were diagnosed simply as "osteosarcoma" of which were diagnosed as grade 4 (highest grade; of these, two patients died of the disease already, and one is alive with metastasis); and the fetal osteoblast (FOB) cell line was a kind gift from Dr. T.C. Spelsberg at the Mayo Clinic. Virtually, all these specimens were collected starting from the early 1960s to early 1980s. Cell culture. Osteogenic sarcoma–derived cell lines (MG63, TE85, SaOS, U2OS, CRL-1423, CRL-1543, CRL-1544, CRL-1547, and CRL-11226) were obtained from the American Type Culture Collection (Rockville, MD), and the fetal osteoblast (FOB) cell line was a kind gift from Dr. T.C. Spelsberg, at the Mayo Clinic. The cell lines were maintained in DMEM/F12 with 10% FCS at 37°C and 5% CO₂ in an incubator.

Isolation of RNA. Total RNA was isolated from cells grown on six-well plates. Cells were washed with PBS and then lysed in Trizol (Life Technologies, Gaithersburg, MD) following the manufacturer’s protocol. RNA samples were treated with DNase to eliminate contaminating genomic DNA (13). Two micrograms of total RNA from each sample were converted to cDNA primed with random primers.

Microarray analysis. Microarray experiments, including initial data analysis, were carried out at the Mayo Microarray Core Facility. Ten micrograms of total RNA isolated from a specimen was used to prepare biotinylated cRNA for hybridization using the Affymetrix protocol (Affymetrix, Santa Clara, CA). Briefly, RNA was first converted to first-strand cDNA using a T7-linked oligo-dT (T7-oligo-dT) primer (Gensen, La Jolla, CA) followed by second strand synthesis (Invitrogen Corp., Carlsbad, CA). The double-stranded cDNA was then used as template for labeling by incubation with biotinylated ribonucleotides (Enzo, Farmingdale, NY). Fifteen micrograms of each labeled and fragmented cRNAs were hybridized to Affymetrix U133A GeneChip (Affymetrix) using standard conditions. Preprocessing of the Affymetrix arrays was carried out using Microarray Suite 5.0. Gene expression intensity for each array was scaled to an average arbitrary value of 1,500 intensity units to allow comparisons across all arrays.

Immunostaining. Paraffin-embedded human osteosarcoma tissues were cut in 5-μm-thick sections and placed on superfrost charged slides. Immunohistochemistry was done essentially as described (14). Briefly, the slides were deparaffinized in xylene (2 × 5 minutes), successively soaked in absolute ethanol and a series of ethanol/water mixtures, and finally rinsed with tap water. Endogenous peroxidase activity was blocked by using 0.3% H₂O₂ in methanol (1:1 v/v). After a tap water rinse, sections were placed into 1 mmol/L preheated (−90°C) EDTA (pH 8) and then steamed for 30 minutes. After cooling in buffered 1 mmol/L EDTA (pH 8) for 5 minutes, the sections were rinsed in tap water and placed in PBS (pH 7.4). Sections were incubated for 5 minutes with DAKO Peroxidase Block (DakoCytomation, Carpinteria, CA) and then incubated with the primary anti-WT1 antibody (Neo-markers, Fremont, CA) at a 1:100 dilution for 30 minutes. The DAKO mouse EnVision+ Horseradish Peroxidase System/AEC+ and the DAKO Autostainer were used for detection. Sections were counterstained with light hematoxylin and then mounted with a coverslip. Basic routine H&E staining (modified Schmidt's hematoxylin) was done for all specimens to ensure tissue quality.

Grading of WT1 immunostaining. WT1 staining was graded from 0 to 4 according to its intensity (0 = no staining, 1 = some (<25%) islands of staining, 2 = homogenous weak staining over 25–50% of the slide, 3 = intermediate intensity and/or islands of intense staining covering 50–75% of the slide, 4 = intense homogeneous staining covering >75% of the slide).

Survival analysis. Only patients with high-grade and/or high grade with metastatic osteogenic sarcoma were subjected to survival analysis. Log-rank statistics was used for all survival curves. Statistical significance was considered as P ≤ 0.05.

Reverse transcription-PCR for WT1 mRNA. The reverse transcription-PCR (RT-PCR) was essentially done as described by Fuchs et al. (15). Briefly, RT-PCR was done in a total volume of 20 μL containing 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 200 μmol/L of each deoxynucleotide triphosphate, 2 μmol/L of each primer, 2 μL of randomly primed cDNA, and 0.5 unit of AmpliTag. Primers from desired segments of WT1 and glyceraldehyde 3-phosphate dehydrogenase mRNAs were included in some PCRs where indicated. PCR were done by first heating the reaction at 94°C for 5 minutes, then cycling the reaction 35 times with condition set as 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute. Five microliters of the amplification products were analyzed by electrophoresis on 2% agarose gel and visualized after staining with ethidium bromide.

DNA sequencing. Automated DNA sequencing of desired PCR-amplified DNA was carried out at the Mayo Molecular Core Facility. In some instances, sequences were obtained for both strands of a template to confirm identity of the DNA.

Results

Strategy to identify survival-associated genes. A direct approach to identify genes associated with survival of patients would constitute comparison of gene expression between samples from patients with good and poor survival outcome. With absent availability of such an ideal sample source, we employed an alternative approach. We speculated that survival is a function of "aggressiveness" (tentatively defined herein as tumor characteristics associated with poor clinical outcome, survival in particular) of a metastatic tumor, which implies that genes associated with very poor survival will be present in tumor specimens that are highly metastatic. We also speculate that aggressiveness of a metastatic tumor is orchestrated by a concerted and/or sequential action of multiple genes, many of which is regulated by "master" genes. Thus, our experimental
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approach consisted of three steps: (a) identifying genes overexpressed in osteogenic sarcoma, (b) evaluate if the candidate gene(s) is also overexpressed in highly metastatic tumor cells compared with poorly metastatic tumor cells, and (c) evaluate pattern of expression of the candidate gene in tumor samples from patients with known survival outcome.

**WT1 mRNA expression is up-regulated in osteogenic sarcoma tumors and in highly metastatic osteogenic sarcoma cells.** Microarray-based analysis of gene expression in 8 normal bone and 10 osteogenic sarcoma biopsy specimens identified hundreds of genes up-regulated in osteogenic sarcoma; nearly 200 of those genes are listed in Supplementary Table S1. Evaluation of the top 100 genes in the list based on available information in the literature suggests that nearly 75% of the genes represent structural (such as proteins associated with the matrix or the cytoskeleton or proteins with apparently no enzymatic activity), whereas the rest (~25%) may have regulatory role (such as transcription factors, cytokines and hormones, and growth factors and their receptors). From this list, WT1 was selected for further evaluation. Furthermore, because WT1 is a transcription factor (a “master” gene) and has been found to be overexpressed in several types of cancers with poor prognosis (16), the gene fulfilled our first criterion for a candidate gene and warranted further investigation. WT1 mRNA expression level was then followed in SAOS, LM8, and 143B cells: one of seven severe combined immunodeficient mice had lung metastasis from SAOS at 6 weeks, whereas 9 of 10 mice had lung metastasis from either LM8 or 143B cells by that time3 by microarray (Supplementary Table S2). Analysis of results from these experiments showed WT1 to be >3-fold up-regulated in osteogenic sarcoma tumors and >2-fold up-regulated in LM8 and 143B cells compared with expression in SAOS cells.

RT-PCR evaluation of WT1 mRNA expression failed to detect the mRNA in total RNA from normal bone (10 of 10; also see Fig. 1A), whereas the mRNA could be identified in a subset of osteogenic sarcoma–derived cell lines (5 of 8; Fig. 1A). These results suggest presence of a mechanism that activates WT1 mRNA expression in a subset of osteogenic sarcoma cells.

**High WT1 expression is associated with poor prognosis of patients with osteogenic sarcoma metastasis.** Immunostaining was done to determine the level of expression of the WT1 protein in a total of 91 osteogenic sarcoma specimens collected before any therapy. Intensity of the immunostain was graded from 0 to 4; an example of the immunostain is presented in Fig. 2. None of the 42 low-grade osteogenic sarcoma specimens stained high for WT1 expression. Twelve of 49 high-grade osteogenic sarcoma specimens stained intensely (24%); all but two of these specimens also had metastasis. Five normal bone samples from as many individuals were also stained, and all of them seem to remain negative for WT1 expression (immunostained sections from three of these normal bones are presented in Fig. 2).

A summary of the relationship between WT1 immunostaining and tumor grade, metastasis, and survival of patients is presented in Table 1. Twelve biopsy specimens of 49 (~25%) high-grade tumors show intense WT1 staining. Thirty-seven patients of 49 total with high-grade lesion at diagnosis developed metastasis (76%); thus, 27% of patients (10 of 37) with metastatic tumors had high WT1 staining. None of the 42 low-grade tumors (including six with metastasis; of these, five patients had good survival outcome) showed high WT1 immunostaining; therefore, all subsequent analyses were done on high-grade tumors only.

To determine if WT1 expression has any relationship on the survival of patients with high-grade osteogenic sarcoma irrespective of metastasis, survival outcome of 12 patients with high WT1 immunostaining was compared with the survival outcome of 37 patients showing little or no WT1 staining. Overall, survival ranged from 4 to 400 months in our cohort of patients (Supplementary Table S3). As presented in the Kaplan-Meier curve in Fig. 3A, patients with high WT1 staining had a statistically significant poorer survival than patients with low WT1 staining (P < 0.03). We also compared survival of patients having high-grade metastatic osteogenic sarcoma with high WT1 staining versus high-grade metastatic osteogenic sarcoma with low WT1 staining and found that mean survival in the first group of patients was 18 ± 12.3 months, whereas survival in the second group of patients was 96 ± 129.3 months (P < 0.0143). Results from this analysis are also presented in Fig. 3B. Thus, it seems that assessment of WT1 expression at diagnosis may have a predictive prognostic value for patients with high-grade metastatic tumor.

**A novel alternatively spliced form of WT1 mRNA is expressed by a subset of osteogenic sarcoma tumor cells.** WT1 mRNA has four alternatively spliced forms (A through D), which differs in their target specificities (17–19). It is important to ascertain whether one or all of these splice variants play a role in the metastasis (survival) of osteogenic sarcoma. The first step towards this goal is to determine which splice variants are expressed in osteogenic sarcoma. RT-PCR was done with a “sense” primer spanning the end of exon 2 and the beginning of exon 3, and an “antisense” primer comprising the beginning of exon 7 and the end of exon 6.

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3 Yuan et al., in preparation.
This primer combination should amplify a 357-bp segment from splice variants B and D (shown by top arrow in Fig. 1A), and a 305-bp segment representing variants A and C (shown by bottom arrow in Fig. 1A). It seems that all the four WT1 splice variants (attempts have not been made to distinguish between variants B and D and between A and C) are expressed in a subset of osteogenic sarcoma–derived cells. None of these isoforms could be amplified by RT-PCR from normal bone (Fig. 1, lanes 7-9). The results, thus, suggest that WT1 mRNA expression is present only in the tumor cells. In addition, we have identified a new splice variant expressed only in a subset of osteogenic sarcoma–derived cell lines (Fig. 1B) but not in normal bone (data not shown). The novel variant, which lacks exons 4 and 5, arises by alternative splicing of the pre-WT1 mRNA (Fig. 4). Whether the novel variant produces any functional protein remains to be established. Taken together, our data suggest that WT1 expression is activated in a subset of osteogenic sarcoma, which is associated with very poor survival.

### Table 1. Relationship between WT1 immunostaining with tumor grade, metastasis, and survival of patient with high-grade osteogenic sarcoma

<table>
<thead>
<tr>
<th>Categories</th>
<th>High-grade osteogenic sarcoma (N = 49)</th>
<th>Metastasis (n = 37)</th>
<th>No metastasis (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1 staining</td>
<td>High*</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Low†</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>Survival outcome</td>
<td>Good‡</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Poor§</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>

**NOTE:** Survival history and WT1 staining intensity of all the 49 samples are presented. The successive numbers in each box were generated manually.

*Staining intensity has been defined in Materials and Methods.

† Survival of ≤60 months has been considered “poor”; survival longer than 60 months has been considered “good.”

### Discussion

A clear observation could be made from the clinical information in our database of patients with osteogenic sarcoma metastasis that patients diagnosed with a metastatic lesion and treated with chemotherapy differ greatly with respect to survival outcome. We explain this observation to be a reflection of “metastatic heterogeneity” and hypothesized that molecular factors other than the ones regulating initiation and progression of metastasis underlie poor survival of patients. We further speculated that patients with the most “aggressive” metastatic lesion (tentatively defined herein as fast-spreading tumors that causes rapid damage to host organ and/or other organs resulting in rapid patient mortality) would experience the worst survival outcome. Based on such a premise, we developed a three-step strategy to identify genes associated with poor survival of patients with osteogenic sarcoma metastasis.

Our results identify WT1 as a gene underlying poor survival of patients with high-grade osteogenic sarcoma especially in the context of metastasis. Several investigators have shown WT1 to be expressed in many cancers other than Wilms’ tumors, including leukemia (20, 21) and cancers of the colon, lung, and breast (22–24). Furthermore, antisense oligo against WT1 mRNA was shown to inhibit growth of WT1-expressing cancer cells (22, 25, 26). Importantly, a high expression of WT1 mRNA was shown to be significantly associated with poor prognosis of patients with breast cancer (21) and leukemias (26, 27). Up-regulation of WT1 expression was also shown to correlate with high tumor stage in testicular germ cell tumors (28). Thus, our results seem to be in accordance with the observation that high expression of WT1 correlates with poor prognosis irrespective of the cancer type. Recently, Ueda et al. (16) reported overexpression of WT1 gene in human bone and soft tissue sarcomas; however, no correlation was made between overexpression of WT1 and any clinical outcome of patients with osteosarcoma.

WT1 was originally identified as a tumor suppressor gene inactivated in Wilms’ tumors, which, like osteogenic sarcoma, primarily affects the children and the young (13). Infants with a predisposition to Wilms’ tumors were found to have germ line mutation in the WT1 gene (17–19, 29). The WT1 gene encodes a Zn finger–containing transcription factor that regulates a
variety of cellular functions by binding to its own promoter and to the promoters of other target genes (19, 30, 31). The gene is known to encode four isoforms generated through alternative splicing; each isoform can regulate a wide spectrum of genes (29, 32). Exon 4 is part of the leucine zipper that is spliced out along with exon 5 in the novel variant that we have identified; thus, due to this potential structural alteration, the novel isoform may have a different set of target genes. Because osteogenic sarcoma tumors apparently express all the splice variants, including the novel one, activation of WT1 expression potentially can create great perturbation in the osteogenic sarcoma tumor cell metabolism.

Recently, Mintz et al. (33) reported the discovery of a set of poor survival-associated genes by employing expression profiling of 15 osteogenic sarcoma samples in each of two groups. One group is classified as Huvos I/II (inferior responders to inductive chemotherapy), whereas the other is classified as Huvos III/IV (very little or no histologic evidence of remaining viable tumor after chemotherapy). Their analysis identified 104 genes; 63 of them up-regulated in Huvos grade 1/2. In addition to the above observation, Ochi et al. (34) and Man et al. (35) have independently identified expression signatures for pre-

![Fig. 3. Kaplan-Meier curve showing relationship between WT1 expression and survival of patients with osteogenic sarcoma. Red lines, samples with low-intensity WT1 immunostaining; green lines, samples with high-intensity WT1 immunostaining.](http://www.aacnjournals.org)

dicting response to chemotherapy. It is highly intriguing that not only these investigators (33–35) did not identify WT1 as a candidate gene; there apparently is no overlap between the gene sets identified by these groups of investigators. We explain this as follows: First, it is known that results of microarray-based experiments can vary widely between laboratories. Second, the analysis criteria used by the three groups of investigators may have excluded WT1 as significantly differentially expressed. Third, only a small subset (12 of 49 high-grade tumors) of our biopsy specimens used for immunostaining (which was our most important criterion for selecting a survival-associated gene) was positive for WT1 expression. Assuming that such a pattern of WT1 expression exists in the 15 Huvos grade 1/2 samples used by Mintz et al. (33), only 3 to 4 of the 15 samples would show high WT1 expression, which could be missed in a background of 12 to 13 low-expressing samples. Finally, the inherent “metastatic heterogeneity” between different sample sets used (by the three groups of investigators and us) may also explain the observation.

We identified WT1 as a survival-associated gene based on our initial comparison between nonmetastatic and metastatic (and between normal versus tumor) osteogenic sarcoma specimens. Subsequently (by immunostaining), however, we find no correlation between WT1 and metastasis per se. We explain this apparent paradox by hypothesizing that WT1 exerts its negative role on survival only in the context of metastasis. This implies that WT1 activation is not a cause of metastasis and will probably have no negative effect in the absence of metastasis. This hypothesis is experimentally testable.

It is necessary to elucidate the mechanism underlying WT1 activation in of osteogenic sarcoma. The WT1 promoter is known to be methylated (36–39); demethylation of the promoter in osteogenic sarcoma is, therefore, a likely mechanism of activation. Given that WT1 is mutated in only ~10% of sporadic Wilms’ tumors (40), it is unlikely that WT1 is mutated in osteogenic sarcoma, but that an epigenetic mechanism underlie its activation. Such a possibility, in fact, is more than likely based on the observation of Ueda et al. (16), who failed to find a mutation in any of the 10 WT1 exons in various sarcoma samples, including osteosarcoma.

We speculate that WT1 does not have a direct role in osteogenic sarcoma metastasis. Recently, it has been reported that at least in the case of breast cancer, separate genes underlie metastasis and poor prognosis (41–43). In the case of osteogenic sarcoma, WT1 may be the first such gene identified to be associated with poor prognosis without being directly associated with metastasis.

Finally, it seems that neither a clear definition of metastasis nor a universally acceptable mechanism underlying the phenomenon is currently available. For example, a recent review on breast cancer presents nine different models of metastasis (44). It is imperative that a clear understanding of the metastatic process is required to evaluate our observation that WT1 expression in osteogenic sarcoma is not associated with metastasis yet correlated with poor survival outcome. We conceptualize cancer and metastasis in the following way: all cancers originate from a single cell that harbors the Knudson’s “two-hit” defect in a “cancer gene” (oncogene/tumor suppressor gene). The functional change in the cancer gene depending on the location of a defect(s) and the developmental stage of the cell harboring the mutated gene determines the future
course of the cancer. Because not all such cancer genes have the same target genes, the course and the outcome of the disease will be a function of which cancer gene initiates the neoplastic process. The propensity of the progeny of the first cancerous cell to acquire further mutation also depends on the nature of the functional defect of the cancer gene. Thus, the functional characteristics of the mutated cancer gene determines whether the progeny of the first cancerous cell will metastasize or not such that defect in a “nonmetastatic cancer gene” will only cause primary tumor, whereas defect in a “metastatic cancer gene” will cause primary tumor with the necessary potential for metastasis. This hypothesis predicts that all progeny cells

![Fig. 4. Sequence chromatogram confirming identity of a novel alternatively spliced WT1 mRNA variant (E, B). Sequence identity of variant D is also shown as a reference (A). Exon junctions (upward arrows). Exons 4 and 5 are spliced out to form the new variant E.](image)

![Fig. 5. Schematic of the necessary to sufficient (NTS) model of osteogenic sarcoma metastasis. NMCG, nonmetastatic cancer gene; MCG, metastatic cancer gene. Primary tumors formed due to abnormality in nonmetastatic cancer gene (probably as in low-grade osteogenic sarcoma) do not (or rarely) metastasize, whereas primary tumors formed due to abnormality in metastatic cancer gene (as in high-grade osteogenic sarcoma) can metastasize through further change. Thus, the two types of primary tumors are not functionally equivalent.](image)
generated from a cancerous cell harboring a deregulated metastatic cancer gene will/can metastasize. However, because it has never been shown that all cells of a particular primary tumor can metastasize (if all cells of a primary tumor can metastasize then, there should not be any primary tumor but tumor can metastasize (if all cells of a particular primary tumor caused by defect in a metastatic cancer gene possess the necessary requirements (first-stage mutations), they are not sufficient for metastasis, and that further changes (second-stage mutations) are required for successful metastasis. This "necessary to sufficient" model (presented in Fig. 5) seems to accommodate both the conventional and the recent alternative hypotheses of metastasis (see ref. 41 for a detailed discussion). Metastatic heterogeneity might be primarily governed by the second-stage mutations; activation of WT1 expression in osteosarcoma specimens might have occurred during or after the second stage mutation in this necessary to sufficient model of metastasis.

References

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