Case Report

Cytogenomic characterization of double minute heterogeneity in therapy related acute myeloid leukemia

Prasad Koduru*, Weina Chen, Barbara Haley, Kevin Ho, Dwight Oliver, Kathleen Wilson

Department of Pathology, and Division of Hematology and Oncology, Department of Medicine, UT Southwestern Medical Center, Dallas, USA

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Breast cancer patients treated with adjuvant chemotherapy regimens containing alkylating agents and anthracyclines are at an increased risk for secondary myeloid malignancies, either acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Complex genomic changes (karyotypes and/or gene amplification) accompany the development of the secondary neoplasms. Here we present a unique case of a breast cancer patient who developed secondary AML within 18 months of treatment with trastuzumab, pertuzumab, docetaxel, carboplatin (TCHP) and radiation. Leukemia cells had catastrophic alterations in chromosomes 8, 11, and 17. Genetic abnormalities in the leukemia cells included amplification of MYC and KMT2A as double minutes, and deletion and mutational inactivation of TP53 Concurrent amplification of different genes at different levels and on different double minutes, we have named “double minute heterogeneity.” Clinically, this case highlights the need to identify genes amplified in secondary myeloid malignancies by cytogenomic microarray (CMA) analysis since these may have therapeutic implications.

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Introduction

Somatic perturbations such as translocation, mutation, deletion, gene amplification and epigenetic modification in proto-oncogenes and/or tumor suppressor genes may result in unscheduled expression of proto-oncogenes, or altered function of tumor suppressor genes, or cause gene overexpression in tumors. Gene amplification is a unique form of genomic instability in the cancer genome and is often considered a biomarker for aggressive disease that is resistant to therapy. While low level amplification is represented by copy number gains (trisomy or tetrasomy), high level amplification may manifest morphologically as a homogeneously staining region (HSR) at a particular chromosome band (intra-chromosomal) or as small light staining paired chromatin structures called double minutes (dmin) scattered in the nucleoplasm [1]. The number of dmin vary from one per cell to several per cell in the same tumor [2]. Dmin and/or HSR have been reported frequently in advanced stage tumors concurrently with complex karyotypes both in diagnostic specimens and in specimens obtained in-therapy or post-therapy [3]. Occasionally dmin are reported as one of the primary genetic aberrations in leukemia [4–6].

Gene amplification is relatively frequent in solid tumors, but it is infrequent in hematopoietic tumors [7]. In AML, or MDS the mechanism for gene gain is most commonly gain of an entire chromosome such as chromosome 8, 11, or 21, whereas KMT2A (MLL), MYC and RUNX1 are amplified infrequently [8–11].

KMT2A encodes a transcriptional coactivator that regulates global gene expression during early embryogenesis and hematopoiesis [12]. Rearrangements (translocations and/or deletions) in this gene are relatively common in hematopoietic neoplasms and more than 130 genomic partners have been identified [13–15]. High level amplification of KMT2A is infrequent but has been reported in AML, MDS and in therapy related B-acute lymphoblastic leukemia [10,16–18]. Amplification of this gene resulting in overexpression has been associated with cell proliferation, increased survival and differentiation arrest [19].

MYC plays a crucial role in signal transduction pathways that promote the cell cycle [20]. MYC is frequently rearranged in B-cell neoplasms, and amplified in solid tumors [21]. Myeloid neoplasms are commonly characterized by trisomy 8 resulting in a copy number gain of MYC, but it is rarely amplified. Ectopic expression of MYC has been shown to result in inducing AML in mice [22]. Nevertheless, whether amplification of MYC or other genes in the amplicon at 8q24.1 contributes to aggressive oncogenic proliferation remains controversial [23].

Earlier studies of dmin in hematological tumors with FISH probes or CMA identified incorporation of MYC or KMT2A or neither in dmin [3]. Here we present a case of secondary AML with dmin some with amplified MYC and the others with amplified KMT2A concurrently, a phenomenon of double minute heterogeneity not reported previously.

* Corresponding author.
E-mail address: prasad.koduru@utsouthwestern.edu (P. Koduru).

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Materials and methods

Morphology and flow cytometry

Peripheral blood (PB) and bone marrow (BM) aspirate smears were stained with Wright-Giemsa. Core biopsy and clot smears were processed and stained with hematoxylin and eosin (HE) and by immunohistochemistry (IHC). Flow cytometric immunophenotyping was performed on the BM aspirate using 10-color (BD FACSCanto™) flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using cluster analysis with Cytofit Classic Software (Leukocyte, CA). Samples were processed and stained with a panel of myeloid, lymphoid and immature cell markers.

Cytogenetics and fluorescence in situ hybridization (FISH)

A BM aspirate obtained at diagnosis and at relapse was harvested for metaphases directly and after culturing for 24 hrs following standard protocols (culturing in RPMI1640 supplemented with 10% FCS and 1% L-glutamine). Metaphase spreads were G-banded, analyzed and karyotypic nomenclature given following ISCN [24].

The probes for 5/5q-, 7/7q-, cep 8, 20q-, KMT2A, and the probes for RUNX1T1/RUNX1, ABL1/BCR, PML/RARA, and CBFB were used to detect MDS and AML related genetic alterations. A dual-color break-apart probe covering MYC, and a tricolor probe for FGFR1 were used additionally to further characterize rearrangements in chromosome 8. Probes for centromere 17 and TP53 were used to establish loss of the short arm of 17. All probes but FGFR1 were purchased from Abbott molecular (Des Plaines IL); FGFR1 probe was purchased from Cytocell (Tarrytown, NY).

Cytogenomic microarray (CMA)

CMA analysis was performed with the DNA extracted from BM using an Agilent CGH+SNP 180k microarray platform (Agilent GXXChip + SNP v1.0 4-plex CGH 180k) according to the manufacturer’s protocol (Agilent Technologies, Santa Clara, CA). Data interpretation was performed using Genophytx software (Perkin Elmer, Waltham, MA). Gain or loss of three contiguous probes was considered as a genomic aberration and annotated according to the human genome build GRCh37/hg19.

Molecular genetics

For TP53 analysis, exons 4–11 were Sanger sequenced using DNA extracted from formalin-fixed paraffin embedded sections of the bone marrow aspirate using a QiAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Amplicons generated using lab-developed M13-tailed PCR primers for each exon were treated with ExoSAP IT (ThermoFisher, Austin, TX) and then bi-directionally sequenced using BigDye v3.1 chemistry (ThermoFisher). Sequencing products were harvested with XTerminator purification reagents (ThermoFisher) and run on an ABI 3730xl DNA analyzer. All DNA sequences were manually reviewed.

Results

The patient was a 63 year old female diagnosed in August 2015 with a HER 2-positive stage II invasive ductal breast carcinoma and treated with TCHP chemotherapy for 6 cycles followed by surgery and breast radiation.

Eighteen months post diagnosis, she complained of fatigue and the exam was remarkable for skin pallor and petechiae. Blood counts revealed a WBC 13,320 with 46% blasts, Hgb 7.6, Hct 23.6, and 29,000 platelets. Bone marrow confirmed an AML with 80% marrow blasts.

Three months later she began 7 + 3 induction therapy with cytarabine and idarubicin. Repeat BM one month later showed persistent leukemia with 80% blasts. A month later tumor lysis syndrome developed with uric acid 8.8 mg and creatinine > 2. Aggressive hydration, allopurinol, rasburicase, transfusions, and leukemia treatment with daconog and hydroxyurea failed to control the WBC. Acidosis, acute kidney injury and respiratory failure developed. Despite antibiotics, antifungals, steroids, fluids, respiratory and dialysis support, the patient status worsened. The family requested transition to comfort care and she expired 21 months from diagnosis.

Morphologic and immunophenotypic findings

A complete blood smear admission at admission showed anemia (Hgb7.6 g/L), leukocytosis (white blood cells 13.28 x 10^9/L) with left-shifted granulocytes, increased myeloblasts (46%), and thrombocytopenia (29 x 10^9/L). BM aspirate and clot section/core biopsy showed hypercellularity (90% cellularity) with a myeloid blast predominance (~80%). Blasts were medium to large in cell size with fine chromatin, prominent nucleoli, variable eosiinophilic granules, and moderate cytoplasm (Figs. 1a and 1b). Immunohistochemical stains showed that blasts expressed MYC (Fig. 1c) and TP53 (Fig. 1d). Flow cytometric immunophenotypic analysis of BM aspirate revealed myeloid blasts with the following immunophenotype: CD4(dim+), CD13(predominantly −), CD14(−), CD15(variably +), CD19(subset partial +), CD33(−), CD34(subset +), CD36(few +), CD38(−), CD45(−), CD56(−), CD64(−), CD79a(−), CD117(variably +), HLA-DR(variably +), CD13(dim+), and TdT(partial +). Given the morphologic and immunophenotypic findings and the history of prior chemotherapy and radiation, a diagnosis of therapy-related AML was rendered.

Cytogenetics

All 20 metaphases analyzed from the diagnostic specimen had an abnormal karyotype constituting two clones, a near diploid clone and a near tetraploid clone; all metaphases had dmin. G-banded karyotype was described as 45XX,add(7)(p22),−8,idic(11)(q11),add(17)(p11.2),del(13)(q11),add(20)(q11.2),del(14)(q32.3),add(15)(q21.3),del(21)(q22.3)(idem)x2[cp5] [Fig. 2]. A karyotype similar to the first clone was obtained from the specimen obtained at first relapse.

FISH results

Since the patient had pancytopenia at presentation, interphase FISH was requested and performed to detect chromosomal abnormalities specific to MDS (deletion 5q or monosomy 5, deletion 7q or monosomy 7, rearrangement or deletion of KMT2A, trisomy 8, deletion 20q), and specific to AML [t(8;21), t(15;17), t(9;22), inv(16)] using standard probes. Probes for 8 centromere (DB822), RUNX1T1 (8q22) and KMT2A (11q23) showed aberrant signal pattern. All other probes showed normal signal pattern. The DB822 probe showed one normal signal and one diminished signal in interphase nuclei (Supplemental Figure 6). This suggested the presence of a partially deleted chromosome 8 centromere in the genome. RUNX1T1 probe showed one signal suggesting loss of one copy of this gene in the leukemia cells. On metaphases the DB822 probe showed one normal signal on the normal chromosome 8 and a diminished signal on the short arm of the add(7)(p22) chromosome; this suggests that the additional material at 7p22 was derived from chromosome 8 [Fig. 3a]. In order to further characterize this abnormal chromosome we hybridized metaphases with FGFR1 probe (8p11.23p11.22) and with RUNX1T1.
(8q22) probe. FGFR1 showed a signal in the middle of the added material at 7p22 (Fig. 3b), whereas the RUNX1T1 had no signal on this chromosome. MYC was also absent on this chromosome (results shown later in Fig. 6b). Thus FISH results for different probes on chromosome 8 suggested complex alterations leading to loss, deletion, and rearrangement of different segments in one homologue.

The KMT2A probe showed amplification and partial deletion in interphase nuclei (Fig. 4a) (Supplemental Figure 1). On metaphases it showed a signal in the pericentromeric region of the idic(11) chromosome and cryptic insertion of the 3′-region into 17p11 (Fig. 4b).

CMA results

Since only some dmin were positive with the KMT2A probe, we performed CMA to detect the origin of the remaining dmin. Genomic CMA profiling identified six clinically significant losses and four clinically significant gains involving chromosomes 8, 11 and 17. The chromosome 8 profile showed four regions of genomic losses and one gain with the following karyotype: arr[GRCh37] 8q11.1q11.21(46923233,50491701)x1, 8q13.2q22.2(70115645_100654309)x1, 8q22.3q24.13(104314771_126137830)x1,8q24.13q24.21 (126170606_130917133) amp, 8q24.21q24.3 (130958847_143242529)x1 (Fig. 5a). This was further confirmed by FISH with MYC break-apart probe (Fig. 6). Two of the regions of loss surrounded the amplified region which was 4.75 Mb and contained MYC (log ratio of >2.5). IHC staining for MYC showed strong positivity suggesting over expression of this gene (Fig. 1c). The chromosome 11 profile showed the following array karyotype: arr[GRCh37] 11p15.5p11.2 (205827_48388756)x3, 11q12.1q23.3(55896790_117046281)x1, 11q23.3(117109943_118471354) amp, 11q23.3(118563828_119924588)x3 (Fig. 5b). In the long arm the large 11q12.1q23.3 deletion was centromeric to KMT2A, and the region of gain at 11q23.3 contained the amplified KMT2A (log ratio of 2.5). The chromosome 17p loss encompassed the entire short arm: arr[GRCh37] 17p13.3p11.2(48858_22192306)x1 (Supplemental Figures 3, 4). From the combined results of G-band karyotype, FISH and CMA the karyotype of the leukemia cells was revised to 45,XX, der(7)(7pter->7p22::8q13->8q11.2::8p10--8pter),der(11)(11pter->11q12.1::11q23.3->11q25::11p11.2->11ppter),add(17)(p11.2),dmin.

Molecular analysis

TP53 sequencing revealed a C141Y mutation (cysteine to tyrosine at amino acid 141) in exon 5, within the DNA binding domain of TP53 (Supplemental Figure 5). This mutation is classified as Pathogenic/Likely Pathogenic in the ClinVar database and was previously shown to decrease expression of p53-regulated genes such as BAX and p21 in osteosarcoma transfection assays [25].
**Discussion**

Genomic regions can amplify in-situ and produce an HSR at the site of amplification. Alternatively a genomic region can excise from the original site, form an episome by fusing the broken ends of the DNA double strand and amplify; this mechanism produces dmin in the nucleoplasm [26–29]. In the episomal model, one allele remains intact and the second allele is deleted and forms an episome. The degree of amplification may vary from a few copies per nucleus to many suggesting continued progression of amplification as the tumor cells multiply. Cases with gene amplification have complex genomic abnormalities and often have loss of TP53 and/or have inactivating mutations in it [30]. Thus, generalized genomic instability induced by prior treatment may be a critical factor in the origin of gene amplification in tumors. The amplified segments, HSR or dmin, possibly harbor genes that confer resistance to standard therapy and promote aggressive clones and hence a poor prognosis. For example, in breast cancer amplification of HER2 is associated with an inferior prognosis [31], and in neuroblastoma amplification of MYCN is associated with advanced...
disease and poor prognosis [32]. Patients with amplified genes may be benefited by agents that target these genes. The dmin may also include proto-oncogenes, whose unscheduled expression is associated with tumorigenesis and/or progression [33,34].

The emergence of acute leukemia after breast cancer treatment has been reported and is usually associated with the use of type II topoisomerase inhibitors or alkylating agents [35–37]. But, patients treated with docetaxel and cyclophosphamide are not at an increased risk for secondary leukemia or MDS [38]. The leukemogenic potential of these two drugs in breast cancer is rarely reported. In the present case, the relatively acute onset of AML18 months after treatment with chemotherapy and radiation for the primary breast cancer mirrors the reports of acute leukemia after type II topoisomerase inhibitors and alkylators.
In hematological neoplasms dmin are reported in de-novo and in treatment related AML. Frequently MYC or infrequently KMT2A are incorporated in the dmin. This is the first report in which both MYC and KMT2A are concurrently amplified in the same tumor but on different dmin. Common features in patients with KMT2A amplification include older age (about 67 years), complex karyotypes, deletion or mutational inactivation of TP53, poor response to treatment and short survival [3,9,10]. Clinical and genetic architecture of leukemic cells of this patient concur with these previous reports.

Catastrophic genetic events

Although the karyotype of the leukemia cells was complex, only three chromosomes (8, 11, and 17) were affected by a multitude of abnormalities that led to rearrangement, or deletion, or amplification of three important genes, namely MYC, KMT2A and TP53. The G-banded karyotype, FISH and CMA results all indicate catastrophic genetic events in one homologue of chromosomes 8 and chromosome 11. Chromosome 8 was monosomic for the most of 8q due to large segmental deletions; two of the four deletions on 8q surrounded the region of gain that contained MYC, which was amplified in the dmin. This suggests that a segment of 8q24.13 excised from its home-site, circularized into an episme, amplified, and overexpressed as detected by the IHC. This is in contrast to one study in which MYC present in dmin was not expressed [23]. Deletion in the long arm of chromosome 8 has been reported in AML patients with MYC amplification [23,41]. Since trisomy 8 in AML provides a mechanistic platform for higher expression of genes on this chromosome [42], the significant oncogenic event in this case was MYC amplification and over expression, which in turn promotes higher proliferation, genomic instability and poor outcome.

Chromosome 11 had multiple aberrations: a gain of the short arm, loss of most of 11q, and cryptic rearrangements involving the 11q23.3 region encompassing KMT2A. One event led to deletion of the 5′-region and cryptic insertion of the 3′-region of KMT2A into the short arm of abnormal 17. In the second event, chromatin containing KMT2A was excised and amplified as dmin. Two distinct tumorigenic perturbations in KMT2A have been implicated in leukemogenesis. The most frequent type of change both in de novo and in therapy related leukemias in children and in adults is rearrangement with different partners in the genome, forming fusion genes and producing fusion proteins. The second type of change, which is rare, is amplification reported in therapy related leukemia [43]. Alterations in KMT2A in secondary leukemia have been attributed to DNA damage induced by either chemotherapy or radiation therapy, or other cellular stress mechanisms [13].

The third event was loss of most of 17p and cryptic insertion of KMT2A into this abnormal chromosome. Loss of 17p lead to loss of one copy of TP53. Although tumor cells had one normal copy of 17, the TP53 allele on this chromosome had mutations which has been reported in hematological malignancies [44,45]. Loss by deletion or mutational inactivation of TP53 is more frequent (up to 40%–60%) in AML with complex karyotypes and gene amplification [3,40,45]. Furthermore, loss of TP53 is documented to lead to genomic instability and gene amplification in tumors [46,47].

In summary, genomically this case had several novel findings not reported previously. The pattern of chromosomal losses and gain around 8q24.13 involving only one chromosome (chromosome 8) suggests excision and episomal amplification of MYC. In contrast, the pattern of losses and gains at 11q23.3 suggests that two mechanisms were involved: intrachromosomal transposition and extra-chromosomal amplification in dmin. Segmental losses and gains in chromosome 8 and in chromosome 11 and concurrent amplification of MYC and KMT2A, and loss of 17p at the diagnosis of secondary AML suggests catastrophic genomic aberrations affecting chromosomes 8 and 11 in a leukemogenic progenitor cell. This insult to the genome may have had occurred during treatment of the previous malignancy. This is the first report of concurrent amplification of MYC and KMT2A on different dmin in a hematologic malignancy. We propose the name “double minute heterogeneity” for this previously undescribed phenomenon. This case also illustrates the need to perform CMA in cases of MDS and/or AML with complex abnormal karyotype and dmin, HSR or ring chromosomes in elderly patients to identify amplified genes. This information is useful in tailoring therapeutic regimen that is effective in disease management.
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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2019.08.001.

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