REVIEW

Double minutes, cytogenetic equivalents of gene amplification, in human neoplasia – a review

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Double minutes are tiny spherical chromatin bodies of a few mega-base pairs of size which are found occasionally in hematopoietic neoplasia and more or less often in human solid tumors. They have been associated with worse prognosis and poor outcome of the malignancies where present. With the beginning era of molecular cytogenetics they could be defined as cytogenetic equivalents of amplified DNA sequences. The identification of involved chromosomal segments and their molecular nature led to the development of molecular genetic techniques for a rapid and reliable detection of prognostically important oncogene amplifications in human tumors and, as a consequence, to gene-targeted therapy.

Key words: double minutes, gene amplification, prognostic value, amplification-targeted therapy.

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INTRODUCTION

Cytogenetics has paved the way to modern tumor genetic analysis. One of the early findings of cancer cytogenetics with far-reaching consequences for oncology was the detection of double minutes in human tumor cells. Double minutes (DM) are tiny spherical chromatin bodies of a few mega-base pairs of size which are observed as pairs in a highly varying number in neoplastic cells. Since their first description[1] they have been found in a vast number of human neoplasias, particularly in solid tumors. Just more than two decades ago it has been shown that DM are a rather frequent finding in human epithelial tumors[2]. However, facing the large number of DM-positive neoplasia cases, it surprises that attempts to identify their chromosomal derivation are relatively rare. Together with the so-called homogenously staining regions (HSR) they have been defined as cytogenetic equivalents of gene amplification since a long time[3-6]. First definition of DM as location of amplified oncogenes[7-10] in neoplasias with poor prognosis has considerably increased the interest of oncologists in these structures. Since oncogene amplifications have become the target for basic therapeutic approaches, molecular techniques of their detection including interphase FISH have more and more replaced the classical cytogenetic detection of DM.

DEFINITION, IDENTIFICATION, AND MOLECULAR STRUCTURE OF DOUBLE MINUTES

First, DM were detected by microscopic analysis of classical chromosome preparations. Their small size, their spherical shape and their characteristic banding pattern (similar to the light G-bands, C-band-negative) were first criteria of their definition[11]. Their early replication pattern was sometimes used as an additional aid[12], but BrDU incorporation revealed that DM are replicated throughout whole S-phase[13,14]. Ultrastructurally they are composed of regularly arranged nucleosome-containing fibers[15]. These are folded to form 30 nm fibers which extend as loops from the chromosome core. DM are lacking both, centromeres[16-18] and telomeres[19,20]. Their number per cell can span a range from one or a few up to hundreds.

First attempts to define the DNA sequences of DM led to genes responsible for therapy resistance. The amplified dihydrofolate reductase (DHFR) gene was the first shown to be overproduced by antifolate-resistant Chinese hamster cells[21]. Its amplification could be demonstrated as soon as the cloned gene cDNA became available for in situ hybridization mapping of the amplified gene on homogenously staining regions[22]. In

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unstably resistant cells the amplified DHFR gene was associated with DM[3]. Later amplified oncogenes could be shown to be located on DM of human cancer cell lines[7-10,23].Therefore, oncogene probes have continued to remain a potent tool of defining newly found DM in human cancers.

A study of DM in a transformed 3T3DM derivative cell line revealed that these DM are a homogenous population of circular molecules, roughly 4 Mb in size, upon which three genes are amplified. One of these is the mdm2 gene[24].

Although the molecular mechanisms of gene amplification are not fully understood[25], several hypotheses have been proposed to explain their generation and their close relation to gene amplification. The so-called episome model, argues that circular molecules (episomes) excised from chromosomes may play an important role in gene amplification and DM formation[26]. These authors prefer a breakage-fusion-bridge model for amplification of genes: it has been suggested that DNA double strand breaks near the amplified segment, incorrect repair, and the resulting "bridge-fusion-bridge" cycle may be involved[27]. Finally these mechanisms result in generation of DM and HSR. A molecular analysis of EGFR amplification in gliomas revealed that "all of the amplicons of a given tumor derive from a single founding extrachromosomal molecule" which was generated by a simple event that circularizes a chromosome fragment overlapping the EGFR gene[28]: "In all cases the fusion of the two ends of this initial amplicon resulted from microhomology-based nonhomologous end-joining. Furthermore, the corresponding chromosomal loci were not rearranged, which strongly suggests that a postreplicative event was responsible for the formation of each of these initial amplicons".

An elegant modern method of the identification of their chromosomal origin is a combination of microdissection and amplification by PCR of the dissected DM-DNA, labelling and using it as a probe for fluorescence in situ hybridization[29,30]. At present large libraries of cosmid probes for many tumor-relevant genes are just available which can be used for FISH on DM. Nevertheless, these methods still are applied relatively rarely for the identification of DM, while those probes at present are intensely used for the detection of amplified oncogenes in interphase nuclei of neoplastic cells[31]. This development was drastically intensified since protein products of amplified oncogenes became a target for new and successful therapeutic approaches[32]. Nevertheless, even this I-FISH screening eventually detects amplification in the form of DM if signals are scattered over the whole nucleus, and in form of chromosome-integrated HSR if the signals are presenting as clusters[33].

Using human neuroblastoma cell lines as model and FISH with a MYCN probe and various chromosome paint probes, Solovei et al[34] could study the topology of DM in interphase nuclei by confocal laser scanning microscopy and quantitative three-dimensional image analysis: "The DM formed dot-like structures in interphase nuclei and were typically located at the periphery of complexly folded chromosome territories. DM noted in the chromosome territory interior were often detected within an invagination of the territory surface". Such a topology was suggested to facilitate access of amplified genes to transcription and splicing complexes that are assumed to localize in the intrachromosomal domain space. It also explains the pattern of distribution of FISH signals of amplified oncogenes detected by modern I-FISH analyses (see below).

DOUBLE MINUTES IN HUMAN LEUKEMIAS AND LYMPHOMAS

Presence of DM, in general, is a rare finding in neoplasias of the hematopoietic system. However, as reviewed by Uehara et al[35], Marinello et al[36] reported the finding of DM in 40 of 320 patients with hematologic diseases. One to three DM per cell in 1% - 100% of the examined cells were found, one DM per cell being most frequent. This review also summarizes some further DM-positive single leukemia cases. Since that time, a number of further reports on DM in hematopoietic neoplasias has been published (table 1), in most cases, however, without a clear definition of the chromosomes of their derivation. Cases with identified DM are collected in table 2.

A recent review on identified DM in AML M2 and M5 cases[49] summarizes 25 cases with amplification of c-myc associated with the DM. Twenty of these cases were classified as FAB M2. In addition, eight cases (3 M5, 2 M4, and 1 each M1, M2, and M6) were reported with amplification of other genes on the DM, (six cases with MLL, one case with c-ETS1, and one case with another gene amplified which was not c-myc and not MLL). Amplification of a contigue containing ETS1, FLI1, SRPR, NFRKB, and KCNJ4 was demonstrated in one AML case[46]. A region of chromosome 19 was involved in DM in two AML M6 cases[39]. In most cases DM carrying amplified oncogenes are part of complex karyotypic alterations and are associated with a poor prognosis.

In very rare cases DM were the sole alteration, or accompanying one single karyotypic change. This is of particular interest with respect to the involvement of DM in the malignant process. Evidently, a characteristic combination in AML M2 is trisomy 4 and DM with c-myc amplification (detected by FISH): 16 cases, so far have been reviewed from the literature[54] mostly found in M2 or M4 ANLL. Apparently this chromosome change modifies the prognostic effect of the DM: Their response to therapy and survival has in some cases been better[49].

TABLE 1. **Double minutes found in human neoplasias (summarized from the available literature; without claim of completeness)**

Hematopoietic neoplasias			Solid tumors		
Neoplasia type	N analysed*	% DM+	Tumor type	N analysed*	% DM+
MDS	236	2.5	Breast cancer	314	18
ANLL	500	4.2	Ovarian cancer	291	29
ALL	376	0.26	Uterine cancer	103	7
CLL	24	8.3	Prostate cancer	177	8.5
MM	254	2.75	Lung cancer	440	20.5
NHL	134	3.7	Pancreatic cancer	101	14
CD^{4+} CD^{56+} DC2 AL	24	4.2	Colon cancer	(52)	44
AL (art)	237	2.1	Bladder cancer	135	14
Various leukemias	376	10.6	Kidney cancer	118	0
Osteosarcomas	146	21	Wilms tumor	167	0
MFH	75	20	Head and neck cancer	201	2.5
Rhabdomyosarcomas	81	15	Malignant melanoma	67	1.5
Chondrosarcomas	106	2	Germ cell cancer	78	4
Lipomas	143	1.5	Astrocytomas	207	10
Liposarcomas	203	9	Gliomas	542	22
Leiomyomas	213	0	Meningiomas	190	1.5
Synovial sarcomas	92	0	PNET	78	5

MDS: myelodysplastic syndrome; ANLL: acute nonlymphocytic leukemia; ALL: acute lymphocytic leukemia; CLL: chronic lymphocytic leukemia; MM: multiple myeloma; NHL: non-Hodgkin lymphoma; AL (art): acute leukemias with acquired Robertson translations; PNET: peripheral neuroendocrine tumor.

TABLE 2. **Identified genomic DNA located on or associated with DM in human hematopoietic neoplasias**

Neoplasia type FAB subtype	Analysed cases	N DM+	Identified DNA content	Ref.
MDS: $^{+}$RA	3	1^{+}	1 DM / 3-13 DM: #11*	37,38
$^{+}$RAEB	1	1^{+}	2-35 DM: # 11	39
$^{+}$RAEBT	1	1^{+}	1 DM: # 8q24* (C8FW)	40
$^{+}$RAEBT	1	1^{+}	ampl. c-myc* (8q24*)	41
$^{+}$RAEBT	2	2^{+}	trisomy 4 + DM: ampl. c-myc	42,43
CML	1	1	numerous DM + Ph'; ampl. abl-bcr	44
CMMoL	1	1	8-40 DM: # 8q24 (no ampl. c-myc)	45
ANLL: $^{+}$M1, M2	2	2^{+}	DM: ampl. c-myc+pvt-1	46,47
$^{+}$M5a	15	1^{+}	5-30 DM: #11* (ampl. MLL)	38
$^{+}$M1,4xM2	5	5^{+}	4x 1DM, / 1x 1-40 DM: 8q24* (ampl. C8FW)	40
$^{+}$M5	6	1^{+}	1-2 DM: #7* (by FISH)	48
$^{+}$M1,20M2,2M3,M5,M6	25	25^{+}	ampl. c-myc	49
$^{+}$M1,M2,2M4,3M5,M6	8	8^{+}	6x ampl..MLL, 1x ampl.c-ETS1, 1x ampl.HTRX-1	49
$^{+}$M1,M3	2	2^{+}	mult. DM: ampl. c-myc	50,51
$^{+}$M2	3	3^{+}	2-64 DM: ampl. c-myc	52
$^{+}$M2	1	1^{+}	2-266 DM: ampl. c-myc	53
$^{+}$M2	3	3^{+}	trisomy 4 + DM: ampl. c-myc	54,55
$^{+}$3M2,3M6	8	8^{+}	1-35 DM: 4x ampl. c-myc; 2x ampl.#19*	39
$^{+}$M2	1	1^{+}	ampl. MLL, ETS1, OPCML, GRIK4; all*	56
$^{+}$M4	1	1^{+}	ampl. c-ETS1	57
$^{+}$n.a.	1	1^{+}	ampl. c-myc*	58
$^{+}$M2	2	2^{+}	1-53 DM/ 1-84 DM associated w. ampl. c-myc	59
$^{+}$M4	19	1^{+}	8-13 DM+r: # 8q24*	60
$^{+}$M6	23	1^{+}	DM: ampl. c-myc*	61
$^{+}$M1,4M2	6	5^{+}	DM: ampl. c-myc*	62,63
$^{+}$4M1	12	4^{+}	DM: ampl. MLL	64
T-ALL	90	5	Ampl. Episomes with NUP214 & ABL-1 fusion	65
NHL:follicular lymphoma	60	1	2-3 DM: # 17*	66

$^{+}$ subtypes of cases with identified DM; * identified by FISH; n.a.: not available; ampl.: amplified; MDS: myelodysplastic syndrome; RA: refractory anemia; RAEBT: refractory anemia with blasts in transformation; CML: chronic myeloic leukemia; CMMoL: chronic myelomonocytic leukaemia; ANLL: acute non-lymphocytic leukaemia; ALL: acute lymphocytic leukaemia; NHL: non-Hodgkin lymphoma; ampl.: amplified; #: chromosome no. N DNA sequences.

As classical cytogenetic analysis supported by specific FISH-techniques still remains the basic genetic examination of leukemia and lymphoma, DM will be easily detected when present. For this reason, and considering the rarity of gene amplification in hematopoietic neoplasias, amplification-directed molecular genetic screening will not play a basic role for delimiting oncogene-targeted therapies in near future.

DOUBLE MINUTES IN HUMAN SOLID TUMORS

Although, in summary, a large number of tumors with DM have been described in the 80s and early 90s, the prevalence of DM-positive cases varies in dependence on the examined tumor type (table 1). Reviewing published cytogenetic analyses of 200 tumors taken directly from patients Benner et al[67] reported presence of DM in 93.5% of these tumors and concluded that DM are the predominant cytogenetic marker for gene amplification.

In epithelial cancers, breast and ovarian cancers, but also various types of lung cancers and colon carcinomas showed the highest percentages of DM-positive cases (breast ca.: 18%; ovarian ca.: 29%, colon cancer ca. 44%). DM are also observed in 20%-30% of small cell lung cancers (SCLC) and 10%-20% of non-small cell lung cancers (NSCLC; 25). However, these percentages varied considerably between the various reports. For instance, Mc Gill et al[68] found DM in 67 of the 76 examined ovarian cancers and reviews variant percentages of DM-positive ovarian cancers from the literature. Our group[2] reported 50 DM-positive cases among 106 examined breast cancers, others found about 10% or even less DM-positive cases.

Testa et al[69] could add first findings on an association of DM with MYC or EGFR amplification in some of the cases of lung cancer. Mitsuuchi and Testa[70] review that MYC amplification occurs in 15%-30% of SCLC and in 5%-10% of NSCLC, figures which well agree with the percentages presented above for DM-positive lung cancers. As most of the relevant studies have been performed before the FISH era, in only few cases an identification of the chromosomal segments the DM are derived from (table 3) was attempted. Amplified oncogenes could be found associated with the DM DNA (see above), but in only very few cases the amplified genes were directly detected on the DM[23]. After the clear definition of the specific amplification of the HER-2/neu (=erbB2) oncogene as a prognostic and predictive factor in breast cancer (but also in other gynaecologic tumors, e.g. ovarian papillary serous carcinomas[94]), rendering this oncogene a target for therapy using a monoclonal antibody[95] genetic analysis turned from screening of DM to I-FISH detection of oncogene amplification.

In *bone and soft tissue tumors* DM apparently are rare events as documented by a series of reviews[96-104]: No DM-positive cases were found among the analysed leiomyomas, Ewing sarcomas, lipomas, synovial sarcomas, dermatofibrosarcoma protuberans and giant cell tumors.

In leiomyosarcomas only reports on small case numbers are available (e.g. 79) which confirm that also in this tumor type DM are rare, but all four DM-positive gastrointestinal stromal tumors collected in a review

TABLE 3. **Identified genomic DNA located on or associated with DM in human solid tumors**

Tumor type	Analysed cases	N DM+	Identified DNA content	References
Epithelial cancers				
Breast cancer	27	1	8q (?) by CGH	71
Ovarian cancer*	1	1	ampl. eIF-5A2	72
NSCLC	100	11	3x ampl. c-myc, 2x ampl. EGFR	70,73
SCLC	13	4	ampl. myc	74
Pleuropulm. blastoma	1	1	ampl. c-myc (by FISH)	75
Thyroid ca.	1	1	# 4 (?)	76
Colorectal ca.	28	18	ampl. c-erbB2 in two of the cases	77
Mesothelial tumors				
Rhabdomyosarcoma	7	3	1-2 ampl. MYCN on DM (by FISH)	78
Leiomyosarcoma	10	2	COAS ampl. on DM (FISH)	79
MFH	11	3	2x ampl. MDM2	80
MPNST	2	1	ampl. COAS + MDM2 on DM	79
Clival chordoma	1	1	# 17 (by FISH)	81
Nerve cell derived tumors				
Medulloblastoma	5	2	Ampl. myc on DM (by FISH)	82
Neuroblastoma	68	8	Ampl. MYCN in 8 cases	83-85,111
	16	?	DM from 16q22q23 (ATBF1); MYCN & MDM2	86,87
Neuroblast. cell lines	2	2	# 2 on DM / ampl. MYCN+DDX1	88-89
Gliomas	134	48	Mostly assoc. with ampl. EGFR, rarely MYCN, GLI, PDGFRA, MET	90,93 111

NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer; *: DM-positive ovarian cancer cell line; MFH: malignant fibrous histiocytome; MPNST: malignant peripheral nerve sheat tumor; ampl.: amplified; #: chromosome no. N DNA sequences.

of Sandberg and Bridge[98] originally had been published as leiomyosarcomas. 19 DM-positive cases were reported from a total of 203 liposarcomas While only 3 of 74 DM carrying tumors belonged to the myxoid and round cell type, 5 of 14 dedifferentiated liposarcomas were DM-positive. Among the 105 reported well-differentiated tumors 8 were DM-positive. In chondrosarcomas DM are also a very rare finding: (two DM-positive cases among 108 reviewed karyotypes). Osteosarcomas, in contrast, show DM more frequently (31 among 146 evaluated cases[102]). In a few cases ring chromosomes and DM were found in identical cells.

As shown in table 3, only few reports have identified the amplified genes located on the DM, i.e. MYCN in three rhabdomyosarcomas, COAS in two leiomyosarcomas and one malignant peripheral nerve sheat tumor, coamlified with MDM2 in the latter case, MDM2 in two malignant fibrous histiocytomas, and sequences from chromosome 17 in a clival chordoma.

Gliomas (without detailed classification according to subtype) rather frequently (more than 20%) are carriers of DM-positive cells. A detailed analysis of oncogene amplification associated with DM revealed the amplified EGFR gene being most frequently located on the DM in human gliomas (table 3). In addition, other amplified genes, e.g. PDGFRA or MET, were found on the DM of single tumors. Amplified MYC was determined by FISH on DM of two *medulloblastomas*.

In neuroblastomas DM are rather frequent, particularly in advanced tumor stages ~30%[105]. In most cases they carry amplified copies of the MYCN gene[106] (table 3). I-FISH analyses of primary *neuroblastomas* detected a pattern of MYCN copy number increase in the interphase nuclei[107] which was very suggestive for being caused by DM. In a neuroblastoma cell line a coamplification of MYCN with DDX1 which is also localized in band 2p24 could be found on the double minutes[89]. More recently, however, neuroblastoma cell lines were identified that, in addition to amplified MYCN, carry DM (or HSR) not harbouring MYCN. On those DM amplified sequences from chromosome 12q13-14 have been identified, and a 30- to 40-fold amplification of the MDM2 gene, located in this region, was found[86]. In a neuroblastoma cell line (SJNB-12) with DM, but no MYCN amplification, the DM were shown to be derived from 16q22-q23 sequences harbouring, among others, the ATBF1 gene, which is an AT-binding transcription factor involved in normal neurogenesis[87].

CLINICAL IMPACT OF DOUBLE MINUTES

Being so closely connected with gene amplification, DM harbour two main gene families which by their amplification in human neoplasias are of considerable clinical impact. Amplified genes localized first on DM were those conferring therapy resistance to the affected tumor cells. In particular, the dihydrofolate reductase which should be inhibited by methotrexate, if present in some hundred (or even thousand) copies, is overexpressed and its excess overcomes the therapeutic action of the cytostatic. As also a number of other resistance-associated genes has been found amplified in neoplastic cells, e.g. by comparative genomic hybridization[108], screening for DM should detect cytogenetic equivalents of those amplifications in several malignancies. Considering the finding of an active decrease of DM by certain substances, as for instance hydroxyurea[67,83,109], new approaches of therapy could be opened by direct deleting those DM which lend therapy resistance to the cells carrying them.

The second group of amplified genes aggregating on DM are certain oncogenes. It has been shown that these oncogenes can be used as a predictor of clinical outcome in human cancer[66]. Just in the early studies on double minutes in primary human tumors it was suggested that their presence in a tumor causes a shorter survival of the affected patient[2]. This first presumption has now been substantiated by a large number of data documenting the prognostic value of DM. In neuroblastomas, for instance, a strong correlation exists between MYCN amplification and stages III and IV. Patients in these stages are known to have a poor prognosis with only 10-30% two-year survival[110].

The findings of the clinical importance of oncogene amplifications were first based on cytogenetic analyses on DM. From those new analytic techniques, e.g. I-FISH[111,112], could be developed which are now basis of the well-known recent amplification-targeted therapy approaches[95,113-118].

Like in solid tumors, in most leukemias DM carrying amplified oncogenes are part of complex karyotypic alterations and are associated with a poor prognosis[49,64]. There have, however, a few cases been found with double minutes as sole chromosomal anomaly which apparently have a better prognosis than cases with complex anomalies[49,64]. Therefore, DM or the gene amplifications located on them cannot be an exclusive factor for clinical development of the disease. The relative rarity of oncogene amplification in hematopoietic neoplasias has so far not led to the same extent of amplification targeted therapy approaches in these diseases like in solid tumors. However, the finding of amplified extrachromosomal episomes in T-ALL containing a fusion of NUP214 and ABL1 could define a new subgroup of T-ALL patients who could benefit from treatment with Imatinib, as this recurrent rearrangement is sensitive to rhis tyrosine kinase inhibitor[65].

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