Unusual Case of Leukemic Mantle Cell Lymphoma With Amplified CCND1/IGH Fusion Gene

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We describe a case of leukemic mantle cell lymphoma (MCL) with complex karyotype and amplification of the CCND1/IGH fusion gene. Testing for the presence of t(11;14), the hallmark of MCL, revealed multiple copies of the fusion signals. We therefore conducted extensive molecular cytogenetic studies to delineate the nature and consequences of such an abnormality. We localized the amplification to the der(14)t(11;14) and to a der(2) chromosome in a form of interspersed chromosome 11 and 14 material. This resulted in high expression of cyclin D1 mRNA and the protein expressed independently of the cell cycle phase. CGH analysis revealed that the overrepresentation on chromosome 11 included chromosomal band 11q23 in addition to the CCND1 locus at 11q13. The band 11q23 harbors the ataxia telangiectasia mutated (ATM) gene recently proposed to be involved in the pathogenesis of MCL with high incidence of deletions in this locus. Using YAC 801e11, containing the ATM gene, we demonstrated several hybridization signals, suggesting that this region also formed part of the amplicon. This case also showed TP53 gene abnormalities: protein expression, monoallelic deletion, and a mutation in exon 5. The clinical course was aggressive, and the patient died within 6 months of presentation. This is to our knowledge the first description of amplification of the CCND1/IGH fusion gene in a human neoplasm, which may have played a role in the fulminating course of the disease in this patient.

Mantle cell lymphoma (MCL) is a distinct entity of mature B-cell neoplasms genetically characterized by the presence of translocation t(11;14) which causes juxtaposition of the cyclin D1 gene (CCND1) located on the long arm of chromosome 11 (11q13) to the long arm of chromosome 14 (14q32) into the immunoglobulin heavy chain locus. This results in the expression of cyclin D1 in the tumor cells, a protein not expressed in the normal counterpart cell (Campo et al., 1999). More than 50% of cases with t(11;14)(q13;q32) show additional and often multiple chromosomal abnormalities, including del(11q), del(13q), rearrangements of 3q, +12, and deletions of 6q, 1p, 9p, and 17p. Translocations involving chromosomes 3, 8, 10, 13, and 17 are frequently encountered in addition to the t(11;14) (Chaganti et al., 2000). Comparative genomic hybridization (CGH) in MCL showed gains at 3q26, 7p15, 8q24, 12q21, and 18q21, and losses at 1q22, 6q21, 9p21, 11q23, 13q14, and 17p13 (Bentz et al., 2000). The incidence of CGH gains was significantly higher in the blastoid form of the disease, particularly at 3q, 7p, 8q, and 12q (Bea et al., 1999).

We present a case of a MCL with a complex karyotype and clustered amplifications of the CCND1/IGH fusion located on the der(14)tt(11;14) and translocated onto chromosome 2. The amplification cluster on der(2) included sequences from cytogenetic band 11q23 as shown by CGH. The ATM (ataxia telangiectasia mutated) gene, recently postulated to play a role in the pathogenesis of MCL through deletions and mutations, is located in this locus. However, we found multiple copies of a YAC containing this gene. As TP53 abnormalities correlate with an aggressive clinical course in MCL, we studied the TP53 gene status in this patient.

A 58-year-old woman presented in August 1999 following a splenic infarct. Physical examination showed massive splenomegaly, and blood test revealed lymphocytosis. She was treated with...
chlorambucil and prednisolone. An infection and pulmonary emboli complicated the clinical course. She was referred to our center in December 1999 for diagnosis and advice concerning management. The white blood cell count (WBC) was then 14.6 × 10^9/L. Treatment with chlorambucil was continued, and splenectomy was suggested as an option if there was no response. The diagnosis of MCL was made with tumor cells expressing CD5 and strong surface immunoglobulins with light chain restriction; CD23 was negative. In January 2000, the disease progressed with rising WBC (187 × 10^9/L) and further spleen and liver enlargement. The patient was febrile, with no obvious infection, despite antibiotic cover. She underwent an emergency splenectomy but died shortly afterwards in metabolic acidosis. Spleen histology was consistent with the diagnosis of MCL. Diagnostic interphase fluorescence in situ hybridization (FISH), carried out as described previously (Gruszka-Westwood et al., 2001), using the t(11;14) detection probe set containing two Locus Specific Identifiers (LSI) IgH / LSI CCND1 (Vysis, Downers Grove, IL), revealed multiple copies of this fusion (Fig. 1A) and prompted detailed molecular cytogenetic studies.
Cytogenetic investigations were performed at stable and progressive stages of the disease according to standard methods. Mitogens used included TPA (phorbol 12-myristate 13-acetate; Sigma, St. Louis, MO) and pokeweed mitogen (Gibco BRL, Gaithersburg, MD). Slides were G-banded according to standard methods and analyzed using the Cytovision System (Applied Imaging Intl., Santa Clara, CA).

Conventional karyotyping revealed a complex karyotype with poor quality of chromosomes. There was a long marker chromosome present in 10/11 abnormal metaphase cells, part of which was identifiable as chromosome 2; however, its detailed assignment was not possible. Other markers were present, and the karyotype at presentation and progression was the same. The result from conventional karyotyping was as follows: 43-45,XX,der(2)add(2)(p23)add(2)(q33)[10], del(3)(p15)[5], del(3)(q12)[5],?add(4)(p15)[2], del(7)(q22)[3], del(7)(q)[11], del(9)[4],add(9)(q21)[3],?add(3)(q21)[2], del(3)(p11)[5],del(3)(q12)[5],?add(4)(p15)[2],del(7)(q22)[3],?add(9)(q21)[3], t(11;14)(q13;q32)[10],?add(19)(q26;p13)[11], del(11)(q10)[4],?add(15)(q10)[4], del(13)(q10)[6],?add(19)(q26;p13),-22[4],+2-5mar[cp11]/46,XX[4].

We then used M-FISH to further the cytogenetic analysis. M-FISH was carried out following the manufacturer’s instructions using the Spectravision probe (Vysis) and analyzed on the Cytovision System. M-FISH analysis disclosed partners for translocations identified by G-banding and explained the derivations of the long marker. The combined conventional and M-FISH karyotype was established as: 43-45,XX,der(2)t(2;13)(p23;q22),del(3)(p23)add(2)(q33)[10], del(3)(p11)[5],del(3)(q12)[5],?add(4)(p15)[2],del(7)(q22)[3],?add(9)(q21)[3], t(11;14)(q13;q32)[10],?add(19)(q26;p13),-22[4],+2-5mar[cp11]/46,XX[4].

FISH was applied for additional characterization of the observed abnormalities. Probes included whole chromosome paints (wcps): 11 and 14 (wcps11 from Cambio, wcps14 from Oncor, Gaithersburg, MD), t(11;14) detection probe set, LSI p53 (Vysis) for detection of TP53 deletion in conjunction with centromere 17 probe (CEP 17, Vysis). Analyses of these experiments were executed using the Vysis Smart Capture system. Metaphase FISH probing with wcps11 and 14 showed the presence of a zebra-like pattern of hybridization consisting of material from these two chromosomes on der(14) and a section of the long marker chromosome. Thus, the final classification of the marker chromosome was as follows: der(2)t(2;13)(?p;?q)t(2;11;9)(?;?;?q1)[16], der(2)t(2;13)(?p;?q)t(2;11;9)(?;?;?q1)[16], der(9)(t(3;9);?;?q1), t(11;14)(q13;q32),i(15)(q10)[6], der(19)t(3;19)(q6;p13),-22[12],+mar.ish der(3)(wcp3)[16][cp16] (Fig. 1B).

Further molecular cytogenetic delineation was conducted with the use of CGH utilizing DNA from cells from the time of acceleration. CGH was performed as described previously (Summersgill et al., 1998) and analyzed using the Vysis Smart Capture System and Quips CGH analysis software. Table 1 shows the summary of chromosomal gains.

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<td>Losses</td>
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<td>der(2)t(2;13)(?p;?q)t(2;11;9)(?;?;?q1)[16]</td>
<td>2p15-25</td>
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<td>2q31-37</td>
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<tr>
<td>3</td>
<td>?add(3)(q21)[2],del(3)(p11)[5],</td>
<td>der(3)(3;7)[14],+mar.ish</td>
<td>3q21-29</td>
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<td>del(7)(q22)[3],del(7)(q)[11]</td>
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<td>9</td>
<td>?add(9)[4],add(9)(q21)[3],?add(9)(q21)[3]</td>
<td>-9[16],der(9)(t;?;?q1)[16]</td>
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<td>13q21-32</td>
<td>13p13-13q14</td>
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<td>15</td>
<td>i(15)(q10)[4]</td>
<td>14q24-32</td>
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<tr>
<td>19</td>
<td>der(19)(q10)(q26;p13)[11]</td>
<td>der(19)(q19)(?q;p1)[16]</td>
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Table 1. Summary of Results Obtained by Different Cytogenetic Methods Displayed by Chromosomes
and losses. Gains were found within the following regions: 3q21-29, 11q12-23, 13q21-32, 14q24-32, and 15q21-26 and losses: 2p15-25, 2q31-37, 3q11-13, 9p24-q22, 11q23-25, 13p13-13q14, and 22q12-13. Overrepresentation at 11q12-23 and 14q24-32 is consistent with amplification of the \( \text{CCND1/IGH} \) fusion gene (Fig. 2). Prompted by the presence of gain at 11q23, \( \text{ATM} \) gene copy number was tested by FISH. CEPH YAC 801e11, mapping to 11q22.3-23.1, containing the \( \text{ATM} \) gene (Stilgenbauer et al., 1999), gave multiple hybridization signals on interphase nuclei, and on metaphases normal signals on chromosome 11 and der(14)t(11;14), but extra hybridization spots on the der(2) (Fig. 1E).

To establish the consequences of \( \text{CCND1/IGH} \) fusion amplification on RNA and protein levels, competitive reverse-transcription polymerase chain reaction (RT-PCR) and flow cytometric protein assays were completed. RNA was extracted using an RNAeasy kit (Qiagen, Chatsworth, CA); RT and competitive RT-PCR were performed as described previously (Cross et al., 1993; Uchimaru et al., 1997). A sample from another MCL patient was included as control. Strong cyclin D1 RNA expres-
sion was detected by RT-PCR. RNA for cyclins D2 and D3 was also observed (Fig. 3). Cyclin D1 protein expression was tested with monoclonal antibody 5D4 (Coulter, Hialeah, FL) using a published method (Elnenaei et al., 2001). The same additional MCL case was included for comparison. Analyses were carried out on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cyclin D1 was strongly expressed in the patient's cells (Fig. 3) throughout the cell cycle and not restricted to the G1 phase.

As TP53 deletion was disclosed, protein expression and direct sequencing were performed on samples from disease progression to search for mutations in this gene. TP53 protein expression was assessed as described previously (Gruszka-Westwood et al., 2001) using monoclonal antibody clone DO1 (Novocastra) and analyzed on a FACScan flow cytometer. TP53 protein expression was detected (Fig. 4B). Direct sequencing of all 11 exons of TP53 was executed by PCR amplification of exonic sequences from genomic DNA followed by fluorescent automated cycle sequencing of both DNA strands (Fig. 4C). Details concerning primers, PCR, and sequencing reaction conditions and analysis are described elsewhere (Gruszka-Westwood et al., 2001). The exon with mutation was resequenced from a different PCR product. A point mutation was detected in exon 5. It was a transversion of thymidine to guanine in codon 173 causing an amino acid change (valine to glycine).

We describe a case of MCL with complex cytogenetics and an amplification of the CCND1/IGH fusion gene. Complex karyotypes are not infrequent in B-cell lymphomas, but an amplification of a fusion gene has not been previously reported. Generally, DNA amplifications were considered a rare abnormality in B-cell lymphomas until the advent of techniques like CGH, which specifically detect imbalances of particular chromosomal regions. Reported gene amplifications in lymphomas include: BCL2 (Monni et al., 1997) and REL proto-oncogene (Houldsworth et al., 1996) in diffuse large cell lymphoma and BCL2 in MCL (Bentz et al., 2000).

Cyclin D1 is a protein product of the PRAD1/CCND1 gene locus on chromosome 11 and, together with its cyclin dependent kinase, is responsible for the transition to the S phase of the cell cycle (Donnellan et al., 1998). Overexpression of cyclin D1 results from gene amplification, presence of translocations (particularly the t(11;14) and its rare variant t(11;22) (Komatsu et al., 1994)), and through disturbance of regulatory mechanisms (Donnellan et al., 1998). Cyclin D1 amplifications have been found in several solid tumors (Donnellan et al., 1998). Recently, cyclin D1 amplification, accompanied by extra copies of chromosome 11, was documented in some cases of multiple myeloma (Hoechtlen-Vollmar et al., 2000).

The formation of t(11;14)(q13;q32) is mediated via the mechanism of V(D)J recombination for the break on chromosome 14 with features of precise cleavage and via double-strand staggered breaks for the breakpoint on chromosome 11. In addition, because point mutations, deletions, and insertions are present in the regions surrounding the breakpoints, template-dependent error-prone DNA synthesis must occur at the time of illegitimate joining. Occasionally, small duplications (1–27 nucleotides) of the BCL1 sequences take place (Welzel et al., 2001).

The presence of t(11;14) leading to cyclin D1 overexpression may not be sufficient for lymphomagenesis, as shown by a transgenic mouse.
experimental model in which mice do not develop spontaneous lymphomas (Lovec et al., 1994). Perhaps this is due to preservation of the periodic oscillation of this G1 protein despite its unscheduled expression in the mantle zone cell, as shown by Lukas et al. (1994a,b). In cell lines and patients with t(11;14) studied by those authors, cyclin D1 expression was restricted to the G1 phase (Lukas et al., 1994a,b). In contrast, cyclin D1 expression throughout the cell cycle was found in some solid tumor cell lines (Juan et al., 1996). Cells from our patient had expression throughout the cell cycle, suggesting that the clone was no longer under control of regulatory mechanisms preserved in patients with classical t(11;14).

The presence of a mutation in the TP53 gene may have been one of the contributing factors towards the aggressive course of the disease. TP53 mutations in exon 5 have been described in aggressive MCL cases (Gandini et al., 1996; Hernandez et al., 1996).

Interestingly, in contrast to recent reports (Stilgenbauer et al., 1999; Schaffner et al., 2000), our patient displayed a high-level amplification at 11q22-23. This locus harbors the ATM gene, which is thought to contribute to the pathogenesis of MCL. However, the YAC probe used contains a large insert (1.2 Mb) from 11q22-23 material, and the ATM gene is 146 kb in size. Thus, it is possible for the ATM deletion to remain undetected by FISH with YAC 801e11 (Stilgenbauer et al., 1999). The use of a smaller probe and mutational studies of the ATM gene would be required to determine whether or not the function of this gene was disturbed.

To our knowledge, there have been no reports of the CCND1/IGH fusion gene being associated with amplification. The formation of gene amplification is underlain by DNA duplication followed by sequential unequal crossing-over increasing the gene copy number. Actual mechanisms involved in this process include extra replication, unequal sister-chromatid exchange, double-stranded breaks at fragile sites, and erroneous repair (Schwab, 1999). Recently, a similar abnormality was described in primary tumor material from two cases of chronic myelogenous leukemia with clusters of BCR/ABL fusion gene amplified and inserted into chromosome 17 (Metzke-Heidemann et al., 2001). Other fusion gene amplifications are described in solid tumors, e.g., the PAX7/FKHR fusion gene, arising as a result of chromosomal translocation t(1;13)(p36;q14) or more rarely amplification of PAX3/FKHR associated with most alveolar rhabdomyosarcomas (Barr et al., 1996; Weber-Hall et al., 1996). PAX7/FKHR is amplified on the double minute chromosomes found in this disorder (Weber-Hall et al., 1996). Amplification arrays of interspersed sequences from two or more chromosomes are described in solid tumors and are frequently associated with formation of ring chromosomes (Naeem et al., 1995; Pedeutour et al., 1995, 1999), but not in hematological malignancies.

Of note also is the agreement of the results obtained by different molecular cytogenetic techniques used in this study and their utility for solving difficult karyotypes (Table 1). Without M-FISH, the identification of the different chromosomes contributing to the long marker chromosome would have been impossible. However, the use of this technique for detection of interspersed amplification clusters is limited, as shown by the M-FISH pattern of the der(2) chromosome, in which only chromosomal material from chromosome 11 was detected. This most likely reflects the fact that the amplified fragment of chromosome 11 was larger than that of 14. As CGH does not detect structural abnormalities but chromosomal imbalances, no abnormalities were detected by this method on chromosomes 4, 7, and 19, which are involved in translocations. Only one of the gains previously reported in MCL was found in this patient—at 3q26 and two of the losses: at 9p21 and 13q14 (Bea et al., 1999; Bentz et al., 2000).

The order of genetic events leading to chromosomal abnormalities is hard to establish, as the cytogenetics at presentation and progression were the same. However, the −9, der(2), t(11;14), der(9), and der(19) were present in all metaphase cells analyzed by M-FISH and in 10/11 by conventional karyotyping, suggesting that these abnormalities happened first.

The patient described here died within 6 months from diagnosis. Median survival for patients with MCL is 2.5–4.5 years (Campo et al., 1999), although the presence of three or more chromosomal aberrations in addition to the BCL1 rearrangement significantly decreases the survival rate (Cuneo et al., 1999). The number of chromosomal changes in the presented case, including a mutation in the TP53 gene, makes it difficult to ascertain which one of these abnormalities was more responsible for the aggressive clinical course.

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