Expression arrays illuminate a way forward for mantle cell lymphoma

Analysis of gene expression profiles in mantle cell lymphomas (MCL) has identified a relatively small group of genes that discriminate MCL from other lymphoma subtypes; a cohort of twenty "proliferation signature" genes predicts patient survival.

Lymphomas and leukemias are generally initiated by founding translocations that deregulate the expression of molecules that control cell cycle progression and differentiation. The hallmark of mantle cell lymphoma (MCL) is the t(11;14) translocation that fuses the immunoglobulin heavy chain enhancer-promoter to the cyclin D1 transcription unit, constitutively activating the cyclin D1 gene (*CCND1*) and disrupting the normal regulation of D-type cyclins by B cell mitogens (see Figure 1). As in other hematopoietic malignancies, additional mutations contribute to a multistep process that not only gives way to overt disease but also determines therapeutic responses to available treatments and attendant prospects for long-term survival.

Rosenwald and coworkers, in this issue of *Cancer Cell* (Rosenwald et al., 2003), describe the use of genomic profiling to examine genes that determine the notoriously variable survival of MCL patients. Lymphochip cDNA microarrays were used to profile gene expression in 101 MCL patients, with an eye toward distinguishing this disease from other lymphoma subtypes. Cyclin D1 was deliberately excluded in order to identify other discriminatory genes and to apply molecular diagnosis to a rare subset of apparent MCL cases ($\sim$9%) that lack cyclin D1 overexpression by PCR analysis. MCL tumor samples with or without cyclin D1 expression were correctly diagnosed based on expression of 42 other MCL "signature" genes. Interestingly, some cases that lacked cyclin D1 expression instead produced high levels of cyclins D2 and D3. Hence, whereas the putative existence of cyclin D1-negative MCL has remained controversial, these results suggest that D1 overexpression can be mimicked at least in part by other D-type cyclins, which presumably drive cell cycle progression in a similar manner.

To identify gene expression profiles that predicted the length of patient survival, a supervised analysis was performed within the 92 MCL cases that were cyclin D1 positive. This fingered 48 genes whose expression correlated with survival duration ($p < 0.001$); all of these genes were more robustly expressed in tumors conveying the worst clinical outcome. Interestingly, a subset of 20 proliferation signature genes was so effective in forecasting survival duration that inclusion of other genes from the original cohort of 48 did not further improve the predictive model. It is curious that no genes that identified patients with the shortest survival were underexpressed. For example, a number of pro-apoptotic genes were reported to be markedly downregulated in MCL compared to nonmalignant lymph node tissue (e.g., *FADD, CASP9, CASP10, RIPK1, DAXX, PDCD1*) (Hofmann et al., 2001), but the findings of Rosenwald and colleagues suggest that their reduced activities are not hallmarks of patients with the worst prognosis.

MCL is essentially an incurable disease with most chemotherapy regimens; success is still measured by prolongation of survival, but not cure (Barista et al., 2001). However, there is some evidence that the intensity of chemotherapy can influence outcome, with dose-intensive cytotoxic chemotherapy coupled with autologous stem cell rescue and total-body irradiation achieving 4-year disease-free survival >70% in patients under 66 years of age (Khouri et al., 1998). In the current study, neither treatment nor patient age were included as covariates in the supervised analysis, but because these patients generally fail therapy, differences in their clinical management may not have significantly biased gene selection. Elucidating the molecular pathogenesis of MCL might ideally identify new targets for the development of more effective agents. Profiling strategies should also help to assess effects of new agents on genes whose expression predicts treatment outcome, thereby serving as a useful tool for drug development and target validation.

Among patients with MCL, the majority of genes that predicted poor long-term survival were those that are expressed at higher levels in dividing cells than in quiescent cells. This proliferation signature correlated directly with increased tumor S phase fractions and mitotic indices and inversely with patient survival. Intriguingly, the proliferation signature segregated different patient subgroups with survival times ranging from less than one year to almost seven years. Expression of cyclin D1 was higher in groups with a relatively poor prognosis, in part due to the preferential production of alternatively spliced, short cyclin D1 mRNA isoforms that exhibit increased stability. Whereas such splice variants cannot be readily discriminated with most cDNA microarrays (e.g., Lymphochip), oligonucleotide arrays can easily be designed to determine the expression level of specific D1 splice variants, thereby offering further insights into the pathogenesis of MCL.

Deletions of the *INK4a/ARF* locus (*CDKN2a*) were detected in about 20% of MCL cases that had a relatively poor prognosis. This locus encodes two distinct tumor suppressors. The $p16^{INK4a}$ protein is an inhibitor of the cyclin D-dependent kinases Cdk4 and Cdk6, preventing their ability to phosphorylate and inactivate the growth-suppressive retinoblastoma protein (RB). The human $p14^{ARF}$ protein, encoded in part from an alternative reading frame (from which it gets its name) of the *INK4a* locus, inhibits the p53 negative regulator, HDM2, to induce a p53-dependent transcriptional program (see Figure 1). Hence, deletion of *INK4a/ARF* in MCL should compromise the tumor-suppressive functions of both RB and p53. Because *INK4a/ARF* deletion and cyclin D1 overexpression were observed to independently contribute to reduced patient survival, these events must cooperate in some way to increase MCL proliferation. How does this work?

Cyclin D-dependent kinases promote S phase entry through two mechanisms (Sherr and Roberts, 1999). The first, now well appreciated, involves inactivation of Rb family proteins by direct phosphorylation. However, the mitogen-dependent accumulation of cyclin D-

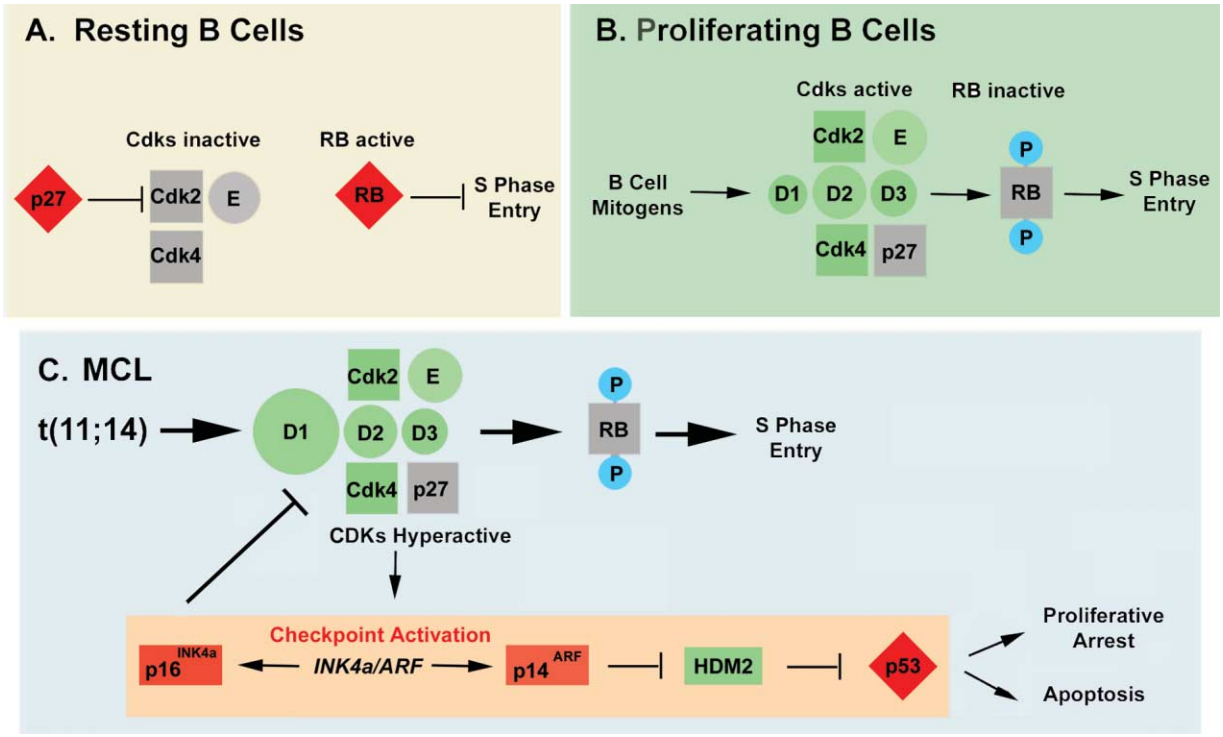

Figure 1. Cell cycle control in normal and malignant B cells

Resting B cells (**A**) do not synthesize D-type cyclins, and cyclin E-Cdk2 complexes are held in an inactive form by $p27^{Kip1}$. B cell mitogens induce synthesis of D-type cyclins (**B**) with cyclins D2 and D3 predominating over D1. Assembly of D-type cyclins with Cdk4 (or Cdk6, not shown) sequesters $p27^{Kip1}$ and facilitates activation of cyclin E-Cdk2. Both Cdks phosphorylate and inactivate Rb family proteins, triggering entry into S phase. In MCL cells bearing the t(11;14) (**C**), cyclin D1 is overproduced and drives S phase entry. In the early stages of disease, development of lymphoma is likely held in check by the two products of the *INK4a/ARF* locus, which antagonize cyclin D1-Cdk4 kinase activity and activate p53 (depicted in the box at bottom). Deletion of the *INK4a/ARF* locus in MCL cancels these checkpoint responses and connotes a poor prognosis.

dependent kinases also sequesters a second class of Cdk inhibitory molecules ($p27^{Kip1}$ and $p21^{Cip1}$) into higher order complexes, thereby preventing these "Cip/Kip" proteins from inhibiting the activity of cyclin E- and A-dependent Cdk2. The catalytic and stoichiometric activities of cyclin D-dependent kinases—namely, RB phosphorylation and titration of Cip/Kip proteins, respectively—work hand in hand to stimulate entry of quiescent cells into S phase. By disrupting cyclin D1-Cdk4 complexes, $p16^{INK4a}$ not only inhibits cyclin D-dependent kinase activity but also mobilizes previously sequestered Cip/Kip proteins, inhibiting Cdk2 and leading to efficient cell cycle arrest. One possibility, then, is that loss of $p16^{INK4a}$ and overexpression of cyclin D1 cooperate to maintain an increased fraction of MCL tumor cells in cycle. This model suggests that progressive increases in the levels of cyclin D-dependent kinase activity, whether due to cyclin D1 overexpression or to $p16^{INK4a}$ loss, determine the probability at which tumor cells enter S phase and subsequently divide.

If this is correct, we might expect that *INK4a* point mutations or silencing, which characteristically inactivate $p16^{INK4a}$ in many other tumor types, would also occur in MCLs. Yet, deletions of *INK4a*, which also affect *ARF*, are the hallmarks of this disease. Minimally, this suggests that loss of $p14^{ARF}$ may contribute to a poor prognosis in MCL, presumably by limiting the activity of p53. In general, *ARF* is induced by oncogenes and, by activating p53, it diverts incipient cancer cells to undergo p53-dependent fates, such as cell cycle arrest or apoptosis. Loss of *ARF* eliminates this cell-autonomous tumor surveillance mechanism and allows activated oncogenes, such as oncogenic Ras or Myc, or possibly cyclin D1, to function unopposed in driving cell cycle progression. If *ARF* loss contributes to MCL, we might not expect to find p53 mutations in those tumors that sustain an *ARF* deletion. However, a few MCL cases had deletions of both the *INK4a/ARF* and *p53* loci, raising the possibility that *p53* loss provides yet additional selective advantages. In mouse B cell lymphomas driven by Myc overexpression, loss of *ARF* accelerates disease progression by primarily limiting Myc-induced apoptosis, whereas loss of *INK4a* disables a cytostatic response to chemotherapeutic agents. Intriguingly, loss of p53 cancels both tumor-protective responses and connotes the worst overall outcome (Schmitt et al., 2002).

Regardless of mechanism, a clear prediction from this work is that inhibitors of cyclin D1 function (reviewed in Senderowicz, 2001) should prolong the life of patients with MCL. However, interfering with cyclin D1-dependent kinase activity (for example, with a small molecule Cdk4 inhibitor) might not be sufficient to arrest disease if the stoichiometric activity of cyclin D1-Cdk4 complexes in sequestering Cip/Kip proteins plays a prominent role. Rather, the ideal drug would be a $p16^{INK4a}$-mimetic—a molecule that disrupts cyclin D1-Cdk4

complexes and releases bound Cip/Kip proteins. Agents acting "upstream" (such as inhibitors of Ras, Raf, and PI3 kinases) can prevent the induction of cyclin D1, limit its stability, or interfere with its assembly with Cdks, and these might prove efficacious. The ultimate goal must be to translate these important new molecular insights into more effective treatment of MCL; until then, we will continue to measure success as extended survival and not cure.

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Oncogene at last—c-Jun promotes liver cancer in mice

c-Jun, a component of transcription factor AP-1, has been known to play an important role in the control of cell proliferation. It was also suspected to be a critical mediator of tumor promotion. In a recent paper in Cell, Eferl et al. have now provided conclusive evidence that c-Jun expression is critical for induction of liver cancer by a classical protocol of tumor initiation—tumor promotion.

The c-Jun protein is a component of transcription factor AP-1 (Angel and Karin, 1991), encoded by the *c-jun* gene (using mouse gene terminology), the cellular homolog of the retroviral *v-jun* oncogene (Vogt, 2001). The discovery fifteen years ago that c-Jun together with c-Fos is a component of AP-1, a transcription factor implicated in the induction of gene transcription by phorbol ester tumor promoters (Angel et al., 1987), generated a great deal of excitement at the time. For once it suggested a biochemical function for c-Jun being one of the first sequence-specific transcription factors found to be encoded by a proto-oncogene. Even more importantly, it suggested that the putative pro-oncogenic function of c-Jun is due to its function at the receiving end of a signal transduction pathway that mediates gene induction by phorbol esters and other tumor promoters. This discovery also provided a molecular mechanism and an explanation for tumor promotion, suggesting that tumor promoters are chemical and physical agents that can activate signaling pathways that stimulate the activity of transcription factors that regulate the expression of genes involved in cell proliferation and neoplastic trasformation. This hypothesis implicated that chronic elevation of c-Jun's expression or activity as brought about by tumor promoters should lead to oncogenic transformation. However, direct genetic evidence in favor of this hypothesis has been lacking. Unlike other mammalian proto-oncogenes, mutations in the *c-jun* locus have not been found in human or murine cancers and overexpression of the normal c-Jun protein does not readily result in transformation of rodent fibroblasts (Shaulian and Karin, 2002). This important deficiency has finally been rectified. Eferl et al. report in the recent issue of *Cell* that a targeted disruption of the *c-jun* gene in mouse hepatocytes does not interfere with normal function, but prevents the emergence of hepatocellular carcinomas in response to a classical model of tumor initiation-tumor promotion (Eferl et al., 2003). These results not only prove that c-Jun is a critical component of the carcinogenic mechanism but also suggest that c-Jun antagonists may be used in chemoprevention of liver cancer, a significant health problem in certain parts of the world.

The acute or chronic loss of hepatic function caused by alcohol, viral infection, or other hepatotoxic drugs can result in severe illness such as fulminant hepatitis, or cirrhosis, and greatly increases the risk for eventual development of hepatocellular carcinoma (Okuda, 2000). Chronic infections with the hepatitis B virus (HBV) and the hepatitis C virus (HCV) represent major risk factors for hepatocellular carcinoma (Okuda, 2000). AP-1 was reported to be activated in both hepatocellular carcinoma and chronic hepatitis (Liu et al., 2002). In vitro studies using liver-derived cell lines have demonstrated rapid activation of AP-1 by HBV or HCV proteins (Kato et al., 2000). Thus, there had been ample reasons to suspect the involvement of c-Jun or other AP-1 proteins in liver cancer.

In addition to c-Jun and c-Fos, AP-1 transcription factors are composed of homo- and heterodimers of basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) subfamilies, all of which recognize the AP-1