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Association of survival and disease progression with chromosomal instability: A genomic exploration of colorectal cancer

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During disease progression the cells that comprise solid malignancies undergo significant changes in gene copy number and chromosome structure. Colorectal cancer provides an excellent model to study this process. To indentify and characterize chromosomal abnormalities in colorectal cancer, we performed a statistical analysis of 299 expression and 130 SNP arrays profiled at different stages of the disease, including normal tissue, adenoma, stages 1–4 adenocarcinoma, and metastasis. We identified broad (> 1/2 chromosomal arm) and focal (< 1/2 chromosomal arm) events. Broad amplifications were noted on chromosomes 7, 8q, 13q, 20, and X and broad deletions on chromosomes 4, 8p, 15q, 17p, 18, 20p, and 22q. Focal events (gains or losses) were identified in regions containing known cancer pathway genes, such as VEGFA, MYC, MET, FGFB, FGFB3, LYN, MMP9, MYBL2, AURKA, UBE2C, and PTEN. Other focal events encompassed potential new candidate tumor suppressors (losses) and oncogenes (gains), including CCDC68, CSMD1, POLR1D, and PMEPAA1. From the expression data, we identified genes whose expression levels reflected their copy number changes and used this relationship to impute copy number changes to samples without accompanying SNP data. This analysis provided the statistical power to show that deletions of 8p, 4p, and 15q are associated with survival and disease progression, and that samples with simultaneous deletions in 18q, 8p, 4p, and 15q have a particularly poor prognosis. Annotation analysis reveals that the oxidative phosphorylation pathway shows a strong tendency for decreased expression in the samples characterized by poor prognosis.

T he development of colorectal cancer from normal epithelial cells through benign adenomas to malignant carcinomas and metastasis is a lengthy, multistep process, involving accumulation of mutations of key regulatory genes (1). Two forms of genetic instability are described in colon cancer. Samples displaying Chromosomal Instability (CIN) comprise 85% of the cases, and the rest display Microsatellite Instability (MIN)—deletions and amplifications of short sequences of nucleotides. MIN tumors are usually euploid, while CIN tumors are aneuploid; these genetic differences are reflected in a different pathological and clinical behavior (2). The authors declare no conflict of interest.

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sample s, see Methods) values obtained from the SNP data. The samples are divided into 3 groups: normal, marked blue in Top color bar (50 samples); near-euploid (green), mainly MIN tumors (25 samples); and aneuploid (red), CIN tumors (55 samples).

We implemented the Genomic Identification of Significant Targets in Cancer (GISTIC) algorithm (9) to identify statistically significant amplifications and deletions. The results displayed in Fig. 1 are for the 55 tumors with high levels of aneuploidy; adding the 25 near-euploid tumors gave nearly identical results. Using the initial set of parameters (referred to as configuration 1; see Methods and SI Methods), we identified 18 broad and 3 focal events (CINons). The location of each CINon, and the corresponding position-dependent statistical significance of the aberration (q-value, see Methods), is shown in Fig. 1B.

We hypothesize that within a CINon, the so-called “driver mutation” (9), which provides a selective advantage to individual tumors, is located in the SNP regions (“peaks”) associated with the minimal q-values. Some CINons have minimal q-values across a large region of the chromosome, making it impossible to locate and identify potential driver mutations (see Fig. 1B). To overcome this problem, we repeated the GISTIC analysis using a different configuration (configuration 2, see Methods and SI Methods). This approach uncovered both additional focal events (see Table 1) that had been subsumed within the signal of the broad amplifications and deletions and narrower q-value minima within these previously identified broad events (Fig. 1C). These additional q-value minima are termed, “peak events” or “peak CINons.” Of importance, several peak CINons identified in this fashion contain previously established cancer pathway genes. Table 1 presents all of the broad, focal, and peak events that we found using both configurations 1 and 2, and the proportion of tumor samples that carry each. Of the 18 broad CINons (see Fig. 1 and Table 1) 8 are amplifications on chromosomes 7p, 7q, 8q, 13q, 20p, 20q, Xp, and Xq, and 10 are deletions on chromosomes 4p, 4q, 6p, 14q, 15q, 17p, 16q, 18q, 20p, and 22q. Most of these broad CINons were previously described (3–5, SI References).

We also found 10 focal CINons; amplifications on 2q, 6p, 8p (part of the 8q broad amplification), 12p, 16q, and 2 on 17q and deletions on 1p, 10q, and 16p. Finally, 17 peak CINons were identified.

### Identifying Correlated Genes

To establish the relationship between copy number changes and gene expression, we identified the U133A expression microarray probe sets (“expressed genes”) that mapped to each CINon detected with the SNP array and computed the Pearson correlation coefficient (PCC) between the associated expression level and copy number (see Methods). A list of significantly correlated “expressed genes” was assembled; the number of such probe sets for every CINon is also presented in Table 1. For some peak CINons, no (or very few) correlated gene was found, but in most cases there were more than 5 and for some broad CINons there were more than 100. Although statistically significant amplifications or deletions that carry no correlated expressed gene are intriguing because they may contain important regulatory elements or express noncoding RNA, we focused our attention on the genes whose mRNA expression was significantly correlated to their copy number.

### Cancer Pathway Genes Significantly Correlated with Broad and Focal Events

CINons: The amplification on 6p contains VEGFA, a growth factor active in angiogenesis, vasculogenesis, and endothelial cell growth (10). 12p contains FGFR6 and FGFR23, members of the fibroblast growth factor (FGF) family, which mediate a variety of cellular responses during embryonic development and in the adult organism and play an important role in tumor angiogenesis (11) (the fact that we did not detect expression of these FGF genes, even though they reside on an amplified segment, may be caused by poor performance of the relevant expression array probe sets). The focal deletion on 10q contains PTEN, a TSG that antagonizes the PI3K–AKT signaling pathway. PTEN, known to be deleted or mutated in many types of cancers (12), is located on 10q23, a genomic region that suffers loss of heterozygosity (LOH) in cancer. Peak CINons contain several known oncogenes: MYC (8q24.21), a transcription factor for growth-related genes, is often amplified and overexpressed in colorectal cancer (13). LYN (8q12.1) is a SRC kinase normally involved in B-cell signaling and cellular proliferation, differentiation, and migration. Interestingly, LYN appears to be a component in the pathway by which CD44 regulates AKT phosphorylation and inhibits apoptosis. Thus amplification of LYN and deletion of PTEN could have synergistic growth-promoting effects on the AKT signaling pathway. Furthermore, upregulation has been associated with resistance to chemotherapy (14, 15). The proto-oncogene MET (7q31.2), a growth factor receptor required for embryonic development, is implicated in tumorigenesis, particularly in the development of invasive and metastatic phenotypes (16). The peak amplification on 20q13.12 contains several oncogenes: MMP9, a matrix metalloproteinase, known to facilitate local tumor spread and metastasis by promoting matrix degradation and cell migration (17); MYBL2, a transcription factor involved in regulation of progression through cell cycle and antiapoptosis (18); UBE2C, a ubiquitin-conjugating enzyme, is significantly overex-
pressed in many different types of cancers, and is associated with the degree of tumor differentiation in different types of carcinomas (19). Another peak amplification, on 20q13.2, contains AURKA, a serine/threonine kinase known as a key regulator of the mitotic cell division process, which has been identified as an oncogene overexpressed in many human cancers (20).

Candidate Cancer Pathway Genes Correlated with Broad and Focal Events. Some of the focal and peak CINons that we found contained candidate cancer pathway genes not previously known to play a role in colon cancer (a list of these correlated genes in these CINons is found in Table S1). One such candidate gene is CCDC68 (18q21.2), also termed cutaneous T cell lymphoma-associated antigen se57–1. The role of CCDC68 in cancer is not known. In colon cancer, CCDC68 is expressed on the cancer cell surface in about 15% of patients (21). In our data this gene is downregulated in 89% of primary tumors (compared with normal tissue) and its expression is highly correlated with the associated gene copy number ($r = 0.51$; $P = 3.6e-5$). These observations suggest that downregulation of this surface protein is an important selective advantage for colon cancer cells and that it should be studied as a potential TSG. CCDC68 is the gene closest to the peak deletion identified on chromosome 18 (Fig. 1C), which is also close to DCC, a known TSG in colon cancer, whose expression level was absent in our samples. CSMD1, located near the peak of the 8p deletion (8p23.2), is not represented on the microarray. Farrell et al. (22) also suggested this gene as a candidate TSG inactivated in colon cancer.

Several candidates are located in amplified regions and are overexpressed. PMEPA1, located on 20q13.31, is an androgen-induced gene expressed in normal prostate tissue. The role of PMEPA1 in cancer is unclear because it shows overexpression in some colon, breast, and ovarian cancers but is underexpressed in prostate cancer. PMEPA1 is known to be induced by the TGF-β signaling pathway, EGF, IGF, and PDGF (23), and it serves to inhibit TGF-β signaling by interfering with TGF-β type I receptor (TGFBR1)-induced R-Smad phosphorylation. Because TGF-β mediates an apoptotic pathway in colon cancer, the effect of PMEPA1 overexpression could be to promote cancer cell growth by fore-
stalling apoptosis. In our data set, PMEPA1 is overexpressed in 84% of the primary tumors (> 2-fold). Expression of PMEPA1 exhibits high correlation with the associated copy numbers \((r = 0.43, P = 9.9e^{-4})\).

The peak region of 13q contains POLR1D, a subunit of both RNA polymerases I and III. RNA polymerase I is involved in the production of 18S, 5.8S, and 28S rRNAs, while RNA polymerase III synthesizes small essential RNAs, such as tRNAs, 5S rRNA, and some snRNAs (24). There is not much information regarding this gene in the literature. POLR1D is overexpressed in 42% of the primary tumors (> 2-fold), showing high correlation between expression and copy number \((r = 0.7, P = 8.6e^{-11})\). Note that in (25) another gene, CDK8, located 1200 kb upstream POLR1D, was associated with this peak amplification.

We found 973 genes the expression of which differs between samples with low and high expression of PMEPA1. They were enriched by genes expressing proteins located in the oxidative phosphorylation pathway. Similar analyses were done for POLR1D and CCDC68 (see Table S2).

**Noncoding RNA.** Some peak and focal CINons contain small noncoding RNA (Table 1). hsa-mir-103–2, identified on the (deleted) peak region of 20p13, was implicated in colon cancer and was found to be downregulated in tumors (26). The peak CINon on chromosome 15 is part of a region that is subjected to genomic imprinting, associated with Prader-Willi syndrome and Angelman syndrome (27). This peak region consists of 76 C/D snoRNAs, organized in 2 large clusters. Currently, these snoRNAs are not known to be related to cancer. Another imprinted locus that consists of large clusters of C/D snoRNAs and microRNAs is located on 14q32 (27). Deletion of C/D snoRNAs could affect methylation of tRNAs or the function of core C/D box snoRNP. Additionally, chromosome 14q is among broad regions that were found to be deleted in colon cancer by others (3, 4) and also by us. Inactivation of imprinted regions requires the silencing of only 1 allele and therefore may serve as an efficient mechanism for inactivating TSGs.

**Derived Copy Number Changes from Expression Data.** One of our principal aims was to search for associations between chromosomal events and clinically relevant features of the tumors, such as survival and progression. To enhance statistical power and reliability, this analysis must be based on as large a number of samples as possible. Because expression data are available for many more samples than are SNP data, it would be useful to impute copy number from expression (28, 29). To establish a reliable procedure, we constructed 2 chromosomal instability maps (see Methods). In both maps (see Fig. S1, for 45 aneuploid tumor samples, which had both expression and SNP data (see Table S3). To minimize variance because of small numbers of genes, we treated only broad CINons and the focal CINon of 1p (because it contained many probe sets). The similarity of the 2 maps is striking, and indeed, the correlations between the entries for each CINon were highly significant \((r > 0.6, P < 0.05\) false discovery rate [FDR]). On the basis of these observations we built an expression-based CINon table (heat map) for all 256 adenoma and tumor samples for which we had expression data (including liver and lung metastasis); this table (heat map) is shown in Fig. 2B for 45 aneuploid tumor samples, which had both expression and SNP data (see Table S3). To minimize variance because of small numbers of genes, we treated only broad CINons and the focal CINon of 1p (because it contained many probe sets). The similarity of the 2 maps is striking, and indeed, the correlations between the entries for each CINon were highly significant \((r > 0.6, P < 0.05\) false discovery rate [FDR]). On the basis of these observations we built an expression-based CINon table (heat map) for all 256 adenoma and tumor samples for which we had expression data (including liver and lung metastasis); this table (heat map) is shown in Fig. 2B and serves as the basis of the analysis described below.

**Association of Aberrations with Survival, Disease Progression, and Other Factors.** We now searched for connections between the different aberrations of Fig. 2B and survival. As expected, there is strong correlation between clinical state and outcome in colon cancer patients: in our data, almost all stage 4 patients had poor outcome (either death of disease or recurrence within 60 months)
and almost all stage 1 and stage 2 patients had good outcome (status other than dead of disease, and recurrence-free interval of >60 months). To evaluate the potential additional value of a chromosome-based classification, we then used a Kaplan-Meier analysis to test for an association between each CINon and outcome (using the imputed CINons of Fig. 2). The lowest p-values (FDR ≤ 10%) belong to 8p, 4p, and 15q (P = 0.008, 0.011, 0.0027, respectively; their Kaplan-Meier plots are shown in Fig. S2. Notably, deletions of chromosomal arms 8p and 4p have previously been related to poor prognosis (30, 31). These same chromosomal abnormalities were also highly correlated with clinical progression as determined by clinical stage 1–4 (P value = 0.0004, 0.0014, and 0.0027, respectively, at FDR ≤ 10% for 8p, 4p, and 15q, respectively). We next wished to learn whether an analysis of outcome in the context of chromosomal amplifications and deletions could provide additional prognostic value to that provided by clinical stage. If so, knowing the specific genes affected by these chromosomal changes could also provide insight into the mechanisms of tumor growth, spread, and resistance to treatment.

Therefore, we used the changes in copy numbers of chromosomal arms to develop a chromosome-based classification of the samples. In the resulting CINon matrices (Fig. 2), the primary tumors and adenomas were divided into 3 groups. Samples of group A (marked blue) have normal copy number of 18q (lost in 71% of the samples); group B (marked green) have deletions in 18q and in either 8p, 4p, or in 15q (but not in all); group C (red) samples have simultaneous deletions in 18q, 8p, 4p, and in 15q; group D (black) have metastases from liver and lung. Group A is composed mostly of adenomas and MIN tumors (see Fig. 2B), known to be mainly euploid (most of these patients had good outcome). Group B is composed of 18 poor and 40 good outcome samples. Group C contains 42 poor and 20 good outcome samples. The Kaplan-Meier plots of groups A + B versus group C is shown in Fig. 2C and D for SNP and expression data, respectively. Whether chromosomal changes are measured in a small group of patients (SNP) or imputed in a larger group (expression) the results are very similar and suggest that coexisting deletions in 18q, 8p, 4p, and 15q is associated with a particularly poor prognosis.

Next we looked for association of the copy number change of each CINon with a variety of other clinical and molecular factors. We separated the samples into 2 groups on the basis of the factor tested (for example, clinical stage or p53 mutation status) and used a t test (on the values from Fig. 2B) to evaluate CINon copy number differences between the 2 groups (at FDR ≤ 10%). Complete results are given in Table S4; here we highlight a few. The most significant copy number differences between adenoma and stage 1 tumors are associated with chromosomal arms 1q, 20q, 2q, and 8q (P < 0.001, FDR = 1%). Consistent with a previous publication (7), 20q amplification is the most frequent, affecting 91% of the tumors (Table 1) and it is even found in some adenomas.

Deletion of 22q was more frequent in stage 2 than stage 3 tumors. Mutant and wild-type p53 samples were distinguished by many amplifications and deletions, the most significant ones being 20q, Xq, 13q, 17p, and 7q (P < 0.001). This is consistent with a view that the p53 mutation is an important step in facilitating chromosomal instability (refs. 5 and 32). Samples harboring mutant KRAS are more likely to display deletion of 20p, while those with a mutation in APC are more likely to have deletion of 18p. Examining the same SNP data, some of us found significant associations between p53 mutation status and copy number changes of 20q, 13q, 20p, and 18q; as well as association of several chromosomal arms with APC mutation but not the association of 20p deletion with kRAS mutational status.

The Oxidative Phosphorylation Pathway Is Related to Survival. Finally, we sought particular molecular pathways that could be affected by the chromosomal amplifications or deletions that we showed to be related to prognosis. We focused on those genes that both mapped to regions affected by copy number change and the expression of which was highly correlated with the chromosomal change, i.e., the “correlated genes” described earlier. Employing the DAVID database (34, 35), we identified 15 pathways that were implicated by these chromosomal aberrations (FDR < 25%) (see Table S5).

Oxidative phosphorylation was the only pathway that had significant association with survival; 23 of the 128 genes assigned by DAVID to oxidative phosphorylation were affected. Of these 23, 14 were downregulated and 9 were upregulated. Six genes are downregulated in the advanced stages of the disease (see Fig. 3A and B). We used SPIN (36) to divide the samples into two groups on the basis of expression of these 6 genes (Fig. 3A). The Kaplan-Meier plot (Fig. 3B) exhibits significant separation of these 2 groups of samples (this was the only one of the 15 implicated pathways that yielded a significant Kaplan-Meier analysis (FDR ≤ 10%; P < 0.01). Fig. 3C shows the samples projected onto the first and second principal component analysis (PCA) from the space spanned by these 6 genes, showing a possible separation between good and poor outcome samples.

The oxidative phosphorylation pathway plays an intriguing role in cancer. In most nontransformed cells, 90% of ATP is produced in the mitochondria through oxidative phosphorylation, while 10% of ATP is attributed to anaerobic glycolysis. This ratio often shifts in tumor cells, where in some systems, more than 50% of the ATP is produced by glycolysis (37). These metabolic changes were identified over 70 years ago (38) and were explained by the hypoxic environment of the tumor. However, it is now understood that some neoplasms shift to glycolysis even under conditions of oxygen sufficiency (37).

HIF1 is a master transcriptional regulator of the adaptive response to hypoxia, which includes upregulation of genes responsible for glycolysis and downregulation of mitochondrial oxidative phosphorylation genes. Normally, the principal mechanism to activate HIF1 is oxidative stress; however, other factors, such as activated oncogenes (Ras, SRC) or loss of TSGs (VHL, PTEN) can activate...
HIF1 even under normoxic conditions (37, 39), as does accumulation of citric acid cycle constituents, succinate, or fumarate (37).

Several explanations have been proffered to explain this “glycolytic shift”: (i) the use of glycolytic metabolites such as ribose or citrate to enhance cell proliferation (37); (ii) high concentrations of lactate create a acidic environment that is advantageous to the process of invasion (40); (iii) decreased mitochondrial function reduces reactive oxygen species (ROS) production; however, the role of ROS in hypoxia-induced death is controversial (37); (iv) the shift to glycolysis also makes the tumor mitochondria less susceptible to permeabilization of the outer mitochondrial membrane, and as a result, less sensitive to apoptosis (40); and (v) the shift helps to conserve O2 for alternative use such as sterol synthesis and oxidative protein folding (37). Our analysis indicates that one mechanism by which the neoplastic cell downregulates oxidative phosphorylation is deletion of the relevant genes and the resulting decrease in the potential of inhibiting the UbcH10 ubiquitin conjugase in human carcinomas of diverse anatomic origin. Cancer Res 69:5275–5283.

Methods
Expression Data. The 299 U133A arrays (see Table S3) were subjected to MAS S, threshold, and log2 transformation (see SI Methods and Fig. S3). Log-expression ratios ERn,s were calculated by subtracting from the log-transformed expression value of probe set s in sample n the median log-transformed expression value of probe set s in the normal colon samples of the same batch (2 batches were identified, because of protocol change). For chromosome X, all normal samples of the same gender and batch were used.

SNP data are composed (see Table S3) of 130 50K XbaI SNP arrays (41). Log-copy number ratios CRn,s were calculated by subtracting from the log-transformed copy number of SNP n in sample s the log-transformed copy number of SNP n in the normal sample. The CRn,s values were smoothed with GLAD (33). See SI Methods for detailed normalization and preprocessing steps.

Identification of CINons. We used GISTIC (9). Q-values for the statistical significance of the copy number of each SNP were calculated for amplifications and deletions separately. CINons were then defined as regions of at least 5 contiguous SNPs with a q-value of <0.25 (for details see SI Methods). Two different configurations of GISTIC were used: configuration 1 aimed at identifying all broad regions and configuration 2 aimed at identifying small CINons (see SI Methods).

Correlation Between Expression and Copy Number. Detailed description is found in SI Methods. For each probe set n, the PCC was calculated between the ERn,s and the CRn,s values of the samples that had both measures of expression and copy numbers (79 samples), and the copy number ratios measured in sample s from SNPs located near the probe set n. Each correlation was assigned with a q-value and FDR of 25% was then used to generate a list of “correlated genes.”

CINon Expression Table. This analysis was limited to CINons that contained many probe sets—i.e., the broad CINons and the focal CINon of 1p. Using the “correlated genes,” we constructed a CINon expression table; for each CINon n and sample s we calculated CEi,n, the median (ERi,n) evaluated for all probe sets n located on CINon n. A CINon copy number table was constructed in the same way, with entry CCi,n derived of CINon n and sample s by taking the median (CRi,n) for all SNPs n that are located on CINon n. The CINon expression table may serve as a predictor of copy number for samples, for which no copy number data were available. To assess the quality of this prediction, PCCs of the CEi,n and CCi,n were calculated for each CINon n over the aneuploid tumor samples. We have used both expression and copy number measurements (45 samples, see Table S3 and Fig. S1).

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16. Rangarajan V, et al. (2004) A CINon copy number table was constructed in the same way, with entry CCi,n derived of CINon n and sample s by taking the median (CRi,n) for all SNPs n that are located on CINon n. The CINon expression table may serve as a predictor of copy number for samples, for which no copy number data were available. To assess the quality of this prediction, PCCs of the CEi,n and CCi,n were calculated for each CINon n over the aneuploid tumor samples. We have used both expression and copy number measurements (45 samples, see Table S3 and Fig. S1).