Frequent Alterations in the Expression of Serine/Threonine Kinases in Human Cancers

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Abstract

Protein kinases constitute a large family of regulatory enzymes involved in the homeostasis of virtually every cellular process. Subversion of protein kinases has been frequently implicated in malignant transformation. Within the family, serine/threonine kinases (STK) have received comparatively lesser attention, vis-a-vis tyrosine kinases, in terms of their involvement in human cancers. Here, we report a large-scale screening of 125 STK, selected to represent all major subgroups within the subfamily, on nine different types of tumors (~200 patients), by using *in situ* hybridization on tissue microarrays. Twenty-one STK displayed altered levels of transcripts in tumors, frequently with a clear tumor type-specific dimension. We identified three patterns of alterations in tumors: (*a*) overexpression in the absence of expression in the normal tissues (10 kinases), (*b*) overexpression in the presence of expression by normal tissues (8 kinases), and (*c*) underexpression (3 kinases). Selected members of the three classes were subjected to in-depth analysis on larger case collections and showed significant correlations between their altered expression and biological and/or clinical variables. Our findings suggest that alteration in the expression of STK is a relatively frequent occurrence in human tumors. Among the overexpressed kinases, 10 were undetectable in normal controls and are therefore ideal candidates for further validation as potential targets of molecular cancer therapy. (Cancer Res 2006; 66(16): 8147-54)

Introduction

The completion of the sequencing of the human genome has revealed that up to ~20% of the human genes encode proteins involved in signal transduction. Among these, there are >500 protein kinases (1) that exert specific and reversible control on protein phosphorylation. Perturbation of kinase signaling, by mutations or other genetic alterations, results in their deregulated expression/activity, and it is often associated with malignant transformation. Protein kinases can be subdivided into tyrosine or serine/threonine specific based on their catalytic specificity. Tyrosine kinases, which include receptor tyrosine kinases, activate numerous signaling pathways, leading to cell proliferation, differentiation, migration, and metabolic changes (2). Moreover, enhanced tyrosine kinase activity is the hallmark of a sizable fraction of cancers as well as other proliferative diseases (3). Serine/threonine kinases (STK) also play a paramount role in cellular homeostasis and signaling through their ability to phosphorylate transcription factors, cell cycle regulators, and a vast array of cytoplasmic and nuclear effectors (4). STK have been implicated in human cancer as well. In particular, the misregulation of kinases belonging to the mitogen-activated protein kinases (MAPK), Aurora/Ipl1p-related kinase, and Polo-like kinases (PLK) families has been associated with tumor growth, metastasis, and poor clinical outcome (5–7).

Kinases constitute an extremely attractive target for therapeutic intervention in cancer, because extensive knowledge on how to inhibit them with small molecules is available. Indeed, a variety of kinase inhibitors have been developed (8), and some of them have found application in cancer therapy. Paradoxically, our ability to interfere with kinases is more advanced than our detailed knowledge of which cancer harbors alterations of which kinase(s). This is especially true for STK, whose alterations in human cancers have received comparatively lesser attention vis-a-vis tyrosine kinases. In recent years, many high-throughput strategies have been exploited to implicate kinases in the initiation and progression of cancer either by searching for activating mutations (9–11) or by identifying misregulated expression in gene profiling experiments (see, for instance, among many, refs. 12, 13). However, to our knowledge, a systematic analysis of the expression of STK in normal and neoplastic human tissues has not been done thus far. In the present study, we analyzed the levels of expression in cancer tissues of a set of 125 STK, including members of all the major branches of the subfamily, by using a high-throughput approach, *in situ* hybridization on tissue microarrays. We report frequent overexpression of STK in some types of cancer. Our findings highlight a major role for STK in cancer and identify possible targets for therapeutic intervention in certain types of cancer.

Materials and Methods

cDNA templates and probe preparation. cDNAs or expressed sequence tag clones were provided by Sugen/Pharmacia (South San Francisco, CA). BLAST searches were done to identify the most specific 300-bp regions in each clone, and specific primers, flanked by T3 and T7 RNA polymerase promoters, were designed for each riboprobe. *In vitro* transcription, done with S35-UTP, was followed by 50G Sephadex probe purification and verification of the appropriate size and labeling of the probe by acrylamide gel. Primer sequences are available upon request.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Tissue samples. Formalin-fixed and paraffin-embedded specimens were provided by the Pathology Departments of Ospedale Maggiore (Novara, Italy), Presidio Ospedaliero (Vimercate, Italy), and Ospedale Sacco (Milan, Italy). For the in-depth analyses of three representative kinases, we used specimens from nontreated patients provided by Istituto Europeo di Oncologia (Milan, Italy) and Ospedale Maggiore.

Real-time quantitative reverse transcription-PCR. RNA extracted from frozen tissues was reverse transcribed in a total volume of 50 μL containing 1× Taqman buffer, 5.5 mmol/L $MgCl_2$, 1 mmol/L deoxynucleotides, 2.5 μmol/L random examers, 20 units RNase inhibitors, and 62.5 units MuLv reverse transcriptase at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes.

Primers and Taqman probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). Primer sequences are available upon request. Amplification reactions were done with the Universal Taqman 2× PCR Mastermix (12.5 μL) in a volume of 25 μL containing 5 μL cDNA plus 300 nmol/L of each primer and 100 nmol/L Taqman probe. All reactions were done in duplicate for 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All reagents were from Applied Biosystems.

Tissue microarray design. For the large-scale screening study, specimens derived from various normal and malignant tumor tissues were analyzed. In breast, colorectal, lung, prostate, and larynx tumors, the "normal" samples were derived from the same patients from whom tumors were obtained; however, normal counterparts were not always available. In non-Hodgkin's lymphomas, the normal counterparts were represented by reactive lymphoid tissues (RLT) of nonneoplastic lymph nodes from different patients. In melanomas, glioblastomas, and sarcomas, normal counterparts were not available. Three tissue microarrays were specifically designed for the screening and prepared as described previously with minor modifications (14). Briefly, two representative normal (when available) and tumor areas (diameter, 0.6 mm) from each sample, identified previously on H&E-stained sections, were removed from the donor blocks and deposited on the recipient block using a custom-built precision instrument (Tissue Arrayer, Beecher Instruments, Sun Prairie, WI). Sections (2 μm) of the resulting recipient block were cut, mounted on glass slides, and processed for *in situ* hybridization or immunohistochemistry.

For the in-depth analysis of three representative kinases, we used three additional tumor type-specific tissue microarrays. Tissue microarray-breast was composed of 92 primary breast infiltrating ductal carcinomas (IDC) and 10 normal breast samples. Tissue microarray-colon included 82 colorectal adenocarcinomas, 28 normal mucosa (from areas adjacent to the tumor areas), 11 hyperplastic polyps, and 28 adenomas (19 without carcinomas and 9 adjacent to infiltrating tumors). Tissue microarray-non-Hodgkin's lymphoma included 10 nonneoplastic RLT and 45 non-Hodgkin's lymphoma of which 14 were of the follicular type and 31 of the diffuse large B-cell type (DLBCL; according to the REAL/WHO classification, 2000).

***In situ* hybridization.** mRNA expression was assessed by *in situ* hybridization using S35-UTP-labeled sense and antisense riboprobes. The *in situ* hybridization was done as described previously (15). Briefly, tissue microarray sections were deparaffinized, digested with proteinase K (20 μg/mL), postfixed, acetylated, and dried. After overnight hybridization at 50°C, sections were washed in 50% formamide, 2× SSC, 20 mmol/L 2-mercaptoethanol at 60°C, coated with Kodak NTB-2 photographic emulsion, and exposed for 3 weeks. The slides were lightly H&E counterstained and analyzed at the microscope with a darkfield condenser for the silver grains.

All tissue microarrays were first analyzed for the expression of the housekeeping gene β-actin to check for the mRNA quality of the samples. Cases showing absent or low β-actin signal were excluded from the analysis (data not shown). In addition, in all cases reported here in which antisense probes yielded positive signals (35 genes), the corresponding sense probe, used as a negative control, did not yield any appreciable signal.

Gene expression levels were evaluated by counting the number of grains per cell and were expressed in a semiquantitative scale (*in situ* hybridization score): 0 (no staining), 1 (1-25 grains; weak staining), 2 (26-50 grains, moderate staining), and 3 (>50 grains, strong staining). *In situ* hybridization scores 2 and 3 were considered to represent an unequivocal positive signal. Scores 0 to 1 were considered as negatives. Misregulation (overexpression or underexpression) was tentatively attributed when differences (only counting positive cases, scores $\geq$ 2) between the tumor and the normal groups were $\geq$20% of the analyzed cases and was further validated by statistical analysis (see below).

Immunohistochemistry. Immunohistochemical staining for ErbB-2 and CRK7 was done using primary anti-HER-2/*neu* (DAKO, Carpinteria, CA; 1:2,000 dilution) or anti-CRK7 (Novus Biologicals, Littleton, CO; 1:1,500 dilution) antibodies, respectively, followed by detection with the DAKO EnVision+ system peroxidase.

Statistical analysis. Concordance among real-time quantitative reverse transcription-PCR (Q-RT-PCR), *in situ* hybridization, and immunohistochemistry was assessed by calculating the Pearson correlation coefficient (r) and χ^2 test.

Significance of overexpression or underexpression and correlations with clinical, histologic, and biological variables were assessed using the Fisher's exact test. Differences were judged significant at confidence levels of $\geq$95% ($P \leq 0.05$).

Results

***In situ* hybridization-tissue microarray validation.** Initially, we investigated whether *in situ* hybridization on tissue microarray could provide reliable (semi)quantitative expression data. We did a parallel analysis of ErbB-2 expression by Q-RT-PCR, *in situ* hybridization, and immunohistochemistry in 39 cases of invasive breast cancers (Supplementary Table S1). By Q-RT-PCR, ErbB-2 mRNA levels ranged from 0 to 362 (mean, 32.5; median, 8). *In situ* analysis (*in situ* hybridization and immunohistochemistry) was done on tissue microarray containing cores from the same 39 patients. To compare results obtained with the three methodologies, we expressed the Q-RT-PCR values as discontinuous groups based on arbitrary thresholds of expression. Data obtained with the three methods were highly concordant (RT-PCR versus *in situ* hybridization, r = 0.906; RT-PCR versus immunohistochemistry, r = 0.909; *in situ* hybridization versus immunohistochemistry, r = 0.910). A total χ^2 test analysis on the frequency of expression values confirmed that there were no significant differences among the three methods used (Supplementary Table S1). Thus, tissue microarray-*in situ* hybridization affords sufficient (semi)quantitative precision for expression analysis on archival specimens.

Analysis of 125 STK by *in situ* hybridization-tissue microarray. One hundred twenty-five STK were selected from all the major branches of the family (ref. 1; Fig. 1; see also Supplementary Table S2; HUGO nomenclature is used throughout this article; in Supplementary Table S2, other common names are also given). Expression was evaluated by *in situ* hybridization on the multitumor tissue microarrays 1 to 3 (Supplementary Table S3) containing ~200 tumor samples frequently along with their matched normal counterparts. Tumors screened included breast, colorectal, lung, prostate, and larynx carcinomas, non-Hodgkin's lymphoma, melanomas, glioblastomas, and sarcomas.

Of the 125 analyzed kinase transcripts, 35 (28%) could be detected, in a reproducible fashion (*in situ* hybridization score $\geq$ 2; see Materials and Methods and Supplementary Table S4), in at least one of the analyzed tissues (Fig. 1). The remaining 90 (72%) genes showed negative or barely detectable signals in all the analyzed tissues likely due to low mRNA abundance. To confirm that lack of detection was due to low mRNA levels, as opposed to failure of the used probes, we selected 10 genes, among the 90 negative ones, to design a second probe in a different region of the transcript. In addition, in this case, no specific signals were detected (data not

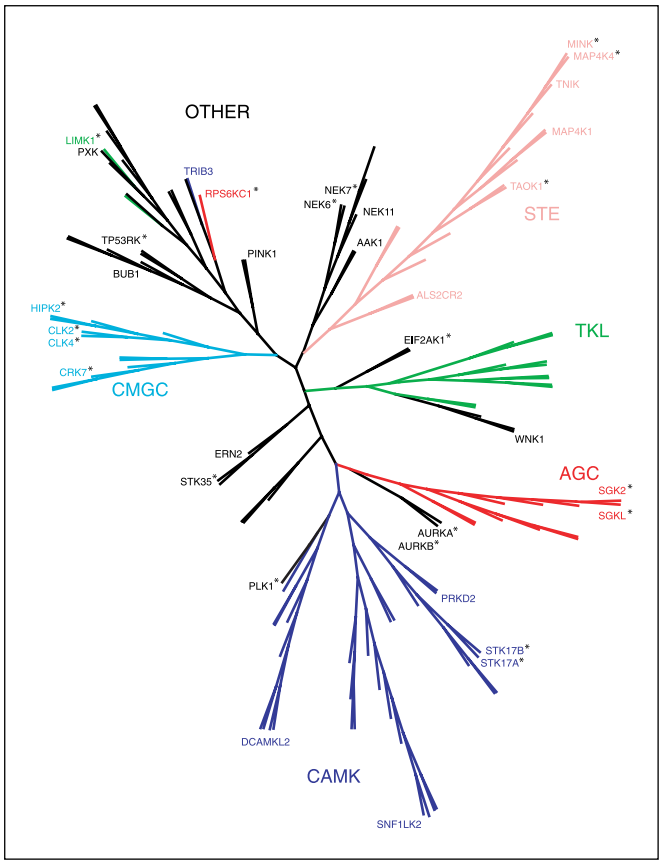

Figure 1. Phylogenetic tree of tested kinases. The kinase domains of the analyzed STK (the complete list is in Supplementary Table S2) were aligned (amino acid level, CLUSTALW), and the alignment was used to construct a phylogenetic tree (CLUSTALW). The 35 STK found expressed by *in situ* hybridization are indicated by their symbols. Kinases found significantly misregulated in tumors are further indicated by an asterisk. Major branches of the family are also indicated with their acronyms. Protein Kinases Group assignment was derived from the kinase database at Sugen/Salk (KinBase; http://kinase.com/kinbase/) and from the Protein Kinase Complement of the Human Genome (1) and is indicated by different coloring. Note that, in some cases, our tree gave different assignments of some kinases with respect to the above Protein Kinases Group assignment.

shown). As a further control, we selected 6 "negative" kinases and analyzed the levels of expression of their mRNAs in breast carcinomas by Q-RT-PCR. As shown in Supplementary Table S5, the 6 kinases showed very low levels of expression when compared with positive controls (2 kinases positive in the *in situ* hybridization assays).

The complete set of results of the screening is shown in Supplementary Table S4. Of the 35 positive kinases, 10 were excluded from further considerations for a variety of reasons. In particular, positive signals for five kinases (TNIK, PXK, BUB1, TRIB3, and ALS2CR2) were too sporadic to allow meaningful analysis. Another three kinases (AAK1, SNF1LK2, and NEK11) were ubiquitously expressed at high levels in 100% of the analyzed cases (tumor and normal samples). The kinase ERN2 was expressed only in colorectum (60-70% of the samples) regardless of the normal or tumor origin. Finally, DCAMLK2 was expressed at high levels exclusively in glioblastomas. However, the lack of matched normal tissues precluded further evaluation of its overexpression.

Alterations in the levels of expression of the remaining 25 kinases, in human cancers, were then evaluated (Figs. 2 and 3 for representative examples). Three distinct subgroups of kinases could be identified (Fig. 2).

(*a*) Up-regulated kinases, class I. These kinases were overexpressed in some human tumors and were not found significantly expressed in any of the normal tissues analyzed (*in situ* hybridization score in normal samples ≤ 1). Ten kinases belonged to this group, and in 29 instances (kinase/individual type of tumor), they were found overexpressed in $\geq$20% of the analyzed tumors (Fig. 2; Supplementary Table S4). In 21 of 29 cases, *P*s for their overexpression were significant ($P \leq 0.05$; Fig. 2; Supplementary Table S5). In the remaining 8 cases, lack of statistical significance is to be attributed to an insufficient number of "normal" samples in the presence, however, of a strong trend (Fig. 2; Supplementary Tables S4 and S6). Of note, all 10 kinases in this group were overexpressed in a statistically significant manner in at least one tumor type (Fig. 2; Supplementary Table S6).

(*b*) Up-regulated kinases, class II. These kinases were overexpressed in some human tumors but also expressed at high levels (*in situ* hybridization score in normal samples ≥ 2) in some normal tissues. Eleven kinases belonged to this group, and in 26 instances

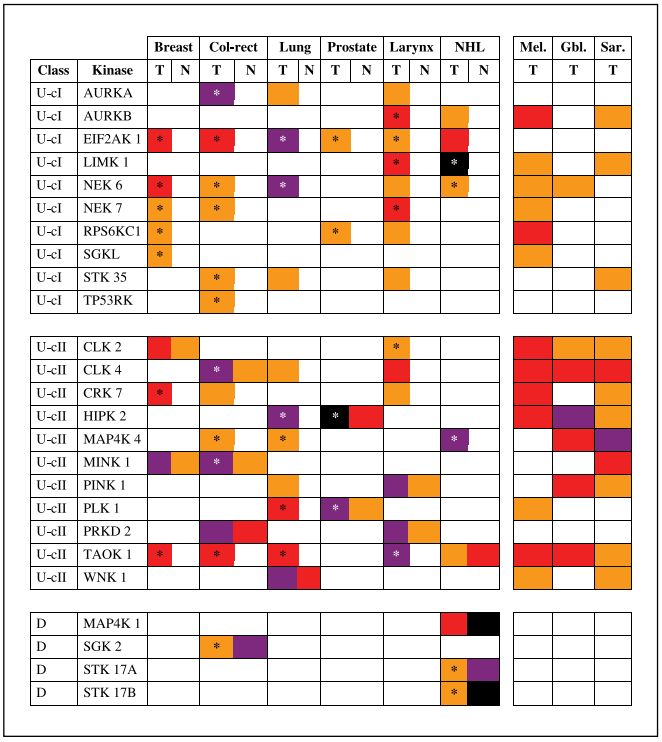

Figure 2. Misregulation of STK in human cancers. Percentage of positive cases (*in situ* hybridization score ≥ 2; see Materials and Methods) in various human tumors (*T*) and matched normal tissues (*N*). Results are expressed in a semiquantitative fashion with the following color code (% of positive cases): *white,* 0-19%; *orange,* 20-39%; *red,* 40-59%; *violet,* 60-79%; *black,* 80-100%. The kinases are shown in three groups to match the three groups identified in the text. In the first group (*U-cI*; up-regulated, class I kinases), all scores are shown. In the second group (*U-cII*; up-regulated, class I kinases), only instances in which there was at least a 20% difference in the number of positive cases (tumor versus normal) are shown (in this group, an white box might either mean 0-19% positive samples or samples with <20% difference tumor/normal). In the third group (*D*; down-regulated kinases) all scores are shown. Details of all scores are in Supplementary Table S4. Asterisks mark those instances in which the misregulation in tumors was statistically significant ($P \leq 0.05$). All statistical analyses are reported in details in Supplementary Table S6. In melanomas (*Mel.*), glioblastomas (*Gbl.*), and sarcomas (*Sar.*), only the percentage of positive cases is reported, because normal tissues were not available. In these cases, *P*s for misregulation could not be determined.

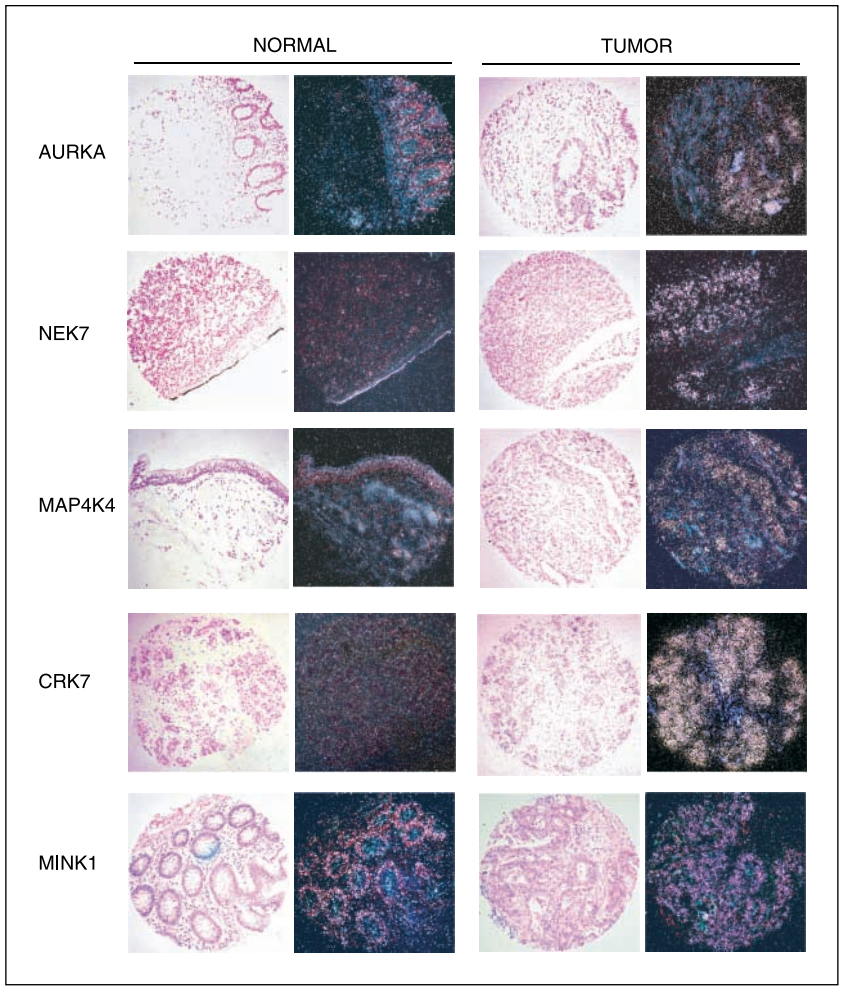

Figure 3. Representative examples of *in situ* hybridization-tissue microarray data. Examples of data summarized in Fig. 2 are shown for normal and tumor tissues. In each pair, *left,* bright field (for morphologic evaluation); *right,* dark field (transcripts appear as bright dots). Shown are examples of up-regulated kinases (class I, AURKA and NEK7; class II, MAP4K4, CRK7, and MINK1). In the examples, AURKA, NEK7, MAP4K4, and CRK7 displayed signals only in tumor cells of colorectal, larynx, lung, and breast carcinomas, respectively. MINK1 was expressed in normal colorectal mucosa and in tumor cells at the same levels (in the shown case).

(kinase/individual type of tumor), they were expressed in ≥20% of the analyzed tumors (*in situ* hybridization scores ≥ 2) and showed ≥20% difference with respect to the normal counterparts (Fig. 2; Supplementary Table S4). In 15 of 26 cases, *P*s for their overexpression were significant ($P \leq 0.05$; Fig. 2; Supplementary Table S6). With the exception of PRKD2, WNK1, and PINK1, overexpression of all other kinases, in this group, reached statistical significance in at least one tumor type (Fig. 2; Supplementary Table S6).

(*c*) Down-regulated kinase. Four kinases belonged to this group, with a clear-cut tissue- and tumor-specific pattern (Fig. 2; Supplementary Table S4). In three instances, down-regulation was observed in non-Hodgkin's lymphomas, and in two of three cases (STK17A and B), it reached statistical significance (Fig. 2; Supplementary Table S6). Some caution is in order in the evaluation of these cases, because the normal tissue employed (RLT) might not necessarily represent the normal counterpart of the tumor target cell. In one case, SGK2, a clear down-regulation in colorectal cancer was observed (Fig. 2; Supplementary Tables S4 and S6). Of note, TAOK1 (belonging to the up-regulated class II kinases) was apparently underexpressed in non-Hodgkin's lymphoma, without reaching, however, statistical significance (Fig. 2; Supplementary Tables S4 and S6).

In conclusion, our data show that 21 of the analyzed kinases were misregulated, in a statistically significant manner, in at least one type of human cancer. It is of note that in all cases analyzed the *in situ* hybridization signals of the overexpressed or underexpressed kinases were associated with the tumor cell component and not with the adjacent or infiltrating stroma (see Figs. 3-5 for representative examples). Thus, alteration in the level of expression of STK is overall a relatively frequent event in cancer.

Extended analysis of representative kinases. If alterations in the expression of STK play a role in the development or in the natural history of certain cancers, then one should expect significant correlations with biologically or clinically relevant variables. As a proof-of-principle validation of this concept, we did an in-depth characterization of three prototype kinases, TP53RK, CRK7, and STK17B, representing each of the three identified classes (see above), in colorectal carcinomas, breast carcinomas, and non-Hodgkin's lymphomas, respectively.

CRK7 (gene aliases: CRKRS, CRKR, and KIAA0904) was investigated on an independent set of 92 breast IDC, and expression data were correlated with clinicopathologic variables. Ten normal breast samples were used as reference for gene expression. No expression was found in normal breast, thus confirming the results of the initial screening (data not shown). Eighteen of 92 (20%) IDC showed high CRK7 levels (*in situ* hybridization score ≥ 2). A significant correlation was found between overexpression and high tumor grade (grade 3; $P = 0.0004$), high proliferative index (Ki-67; $P = 0.009$), negative hormonal status (estrogen receptor,

$P < 0.0001$; progesterone receptor, $P = 0.01$), and positive ErbB-2 ($P < 0.0001$), whereas no correlation was found with pT and nodal status (Supplementary Table S7). As a further validation, we did a parallel analysis of CRK7 expression by Q-RT-PCR, *in situ* hybridization, and immunohistochemistry in breast cancers. The three methodologies were highly concordant (Fig. 4). The fact that CRK7 overexpression could be confirmed also at the protein level, together with the correlation with clinical and pathologic variables, strongly suggests that overexpression of CRK7 marks aggressive breast IDC.

From our initial screening, TP53RK (gene aliases: RP1-28H20.1, C20orf64, Nori-2, Nori-2p, and PRPK) was preferentially up-regulated in colorectal adenocarcinomas. In this type of tumors, it is possible to define progressive stages of cancer development. Thus, we analyzed a colorectal cancer progression tissue microarray containing normal epithelia, hyperplastic polyps, adenomas, and adenocarcinomas (28 normal colorectal epithelia, 11 hyperplastic polyps, 28 adenomas, and 82 primary colorectal adenocarcinomas). All normal and hyperplastic samples as well as 26 of 28 (93%) adenomas were negative. Thirty-eight of 82 (46%) adenocarcinomas showed TP53RK up-regulation (*in situ* hybridization score ≥ 2; Fig. 5*A* and *B*). Within the tumor class, no correlation was found between TP53RK expression and pathologic features, such us grade, local tumor invasion, nodal status, and Dukes stage (data not shown). Importantly, we also showed that TP53RK mRNA levels, detected by *in situ* hybridization, correlated well with those detectable by Q-RT-PCR (Q-PCR versus *in situ* hybridization, $r = 0.90$; Supplementary Fig. S1). Together, all these results implicate the overexpression of TP53RK in the conversion from the premalignant to the malignant state in colon cancer.

Finally, STK17B (gene alias: DRAK2), one the down-regulated kinases, was evaluated on a large set of non-Hodgkin's lymphoma. Nonneoplastic RLT (10 cases) were again found strongly positive for STK17B, which appeared mainly expressed in the follicular centers (Fig. 5*C* and *D*). In 100% of the case of non-Hodgkin's lymphoma of the follicular type, STK17B was also strongly expressed, with a pattern of expression partially recapitulating that observed in RLT. In contrast, only 32% of the non-Hodgkin's lymphoma of the DLBCL type displayed STK17B signals. Statistical analysis confirmed the significant down-regulation of this kinase in DLBCL relative to RLT and follicular type (Fisher exact test, $P = 0.0002$ and $P < 0.0001$, respectively; Fig. 5*C* and *D*). Of note, DLBCL is characterized by a more aggressive behavior and poorer prognosis than follicular type.

Overall, the above data indicate that significant correlations can be found between alterations in the expression of selected STK and relevant biological and/or clinicopathologic variables in human cancers.

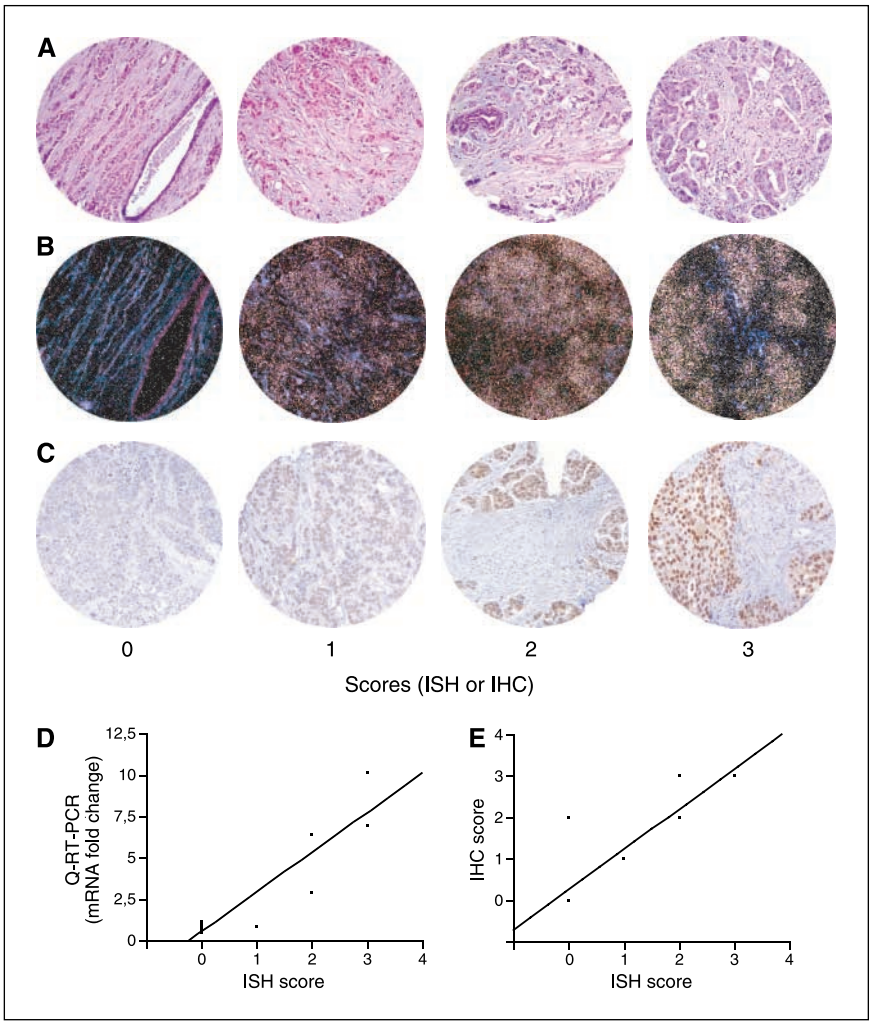

Figure 4. CRK7 expression in breast carcinomas. *A* to *C,* examples of data used for the analyses shown in (*D* and *E*). Four breast carcinomas are shown, displaying different expression levels of CRK7. *A,* H&E stain; *B, in situ* hybridization (*ISH*); *C,* immunohistochemistry (*IHC*). Analyses were done on serial tumor sections. CRK7 protein overexpression (immunohistochemistry) was evaluated by assessing the intensity of nuclear staining in the tumor cells and scored as follow: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. *In situ* hybridization scoring criteria are described in Materials and Methods. *D* and *E,* correlation between CRK7 expression levels measured by *in situ* hybridization and Q-RT-PCR (*D*) or *in situ* hybridization and immunohistochemistry (*E*). Comparative analysis was done on 13 breast tumor samples. Q-RT-PCR results are normalized to an endogenous reference (18S) and expressed as relative levels of CRK7 mRNA, referred to a sample, chosen to represent 1× expression of this gene. Pearson correlation, Q-PCR versus *in situ* hybridization, $r = 0.92$; *in situ* hybridization versus immunohistochemistry, $r = 0.89$. Note that in (*E*) there appears to be fewer samples than those actually tested ($n = 13$), because in many cases there were identical *in situ* hybridization/immunohistochemistry scores (e.g., ISH-0/IHC-0 = 7 samples, represented by a single dot).

Discussion

By doing a wide survey of human tumors, we identified 21 kinases whose levels of expression were significantly misregulated in several types of cancer. *In situ* hybridization-tissue microarray has been proven to be a reliable methodology to conduct this kind of studies. Although more sensitive methods, such as Q-RT-PCR, could result in a higher rate of detectable genes, we believe that the advantage of *in situ* hybridization-tissue microarray is 2-fold. (*a*) The higher sensitivity threshold of *in situ* hybridization-tissue microarray turns out to be an advantage by allowing the identification only of clearly misregulated genes and missing minor fluctuations that might reach statistical significance and still remain of uncertain biological significance. (*b*) *In situ* hybridization allows precise identification of the cellular origin of the signals and discrimination of signals associated to the tumor component from those associated to the tumor microenvironment, an issue of obvious relevance for both the understanding of cancer and the design of molecular therapies. Some caution, however, must be exerted in generalizing the biological significance of overexpression or underexpression detected at the mRNA level in the absence of protein expression data. In this study, we could establish good correlation between mRNA and protein levels in the sole case, CRK7, analyzed as a proof of principle. Clearly, more studies will be needed to establish whether, in all the cases reported here, overexpression is also detectable at the protein level, a necessary prerequisite to draw firm biological conclusions.

Perturbations of several kinases identified in this study were already reported in human tumors by (*a*) classic high-resolution studies (AURKA, AURKB, MAP4K4, LIMK1, and PLK1; references are in Table 1), (*b*) high-throughput expression profile studies (AURKB, CLK2, EIF2AK1, HIPK2, LIMK1, MAP4K4, NEK6, and PLK1; references are in Supplementary Table S8), or (*c*) mutational studies of human cancers (EIF2AK1, NEK7, PLK1, RPS6KC1, SGKL, and TAOK1; references are in Supplementary Table S9). However, for several of the misregulated kinases (CLK4, CRK7, MINK1, SGK2, STK17A, STK17B, STK35, and TP53RK), we report here, to the best of our knowledge, the first evidence of deregulation in human cancers. The predominant alteration detected was overexpression, a finding in line with the known or suspected function of many signaling kinases as positive effectors in proliferation and proliferation-related processes. Indeed, a Gene Ontology analysis (summarized in Table 1 and presented in an extended form in Supplementary Table S10) revealed that most of the overexpressed kinases have established or putative functions

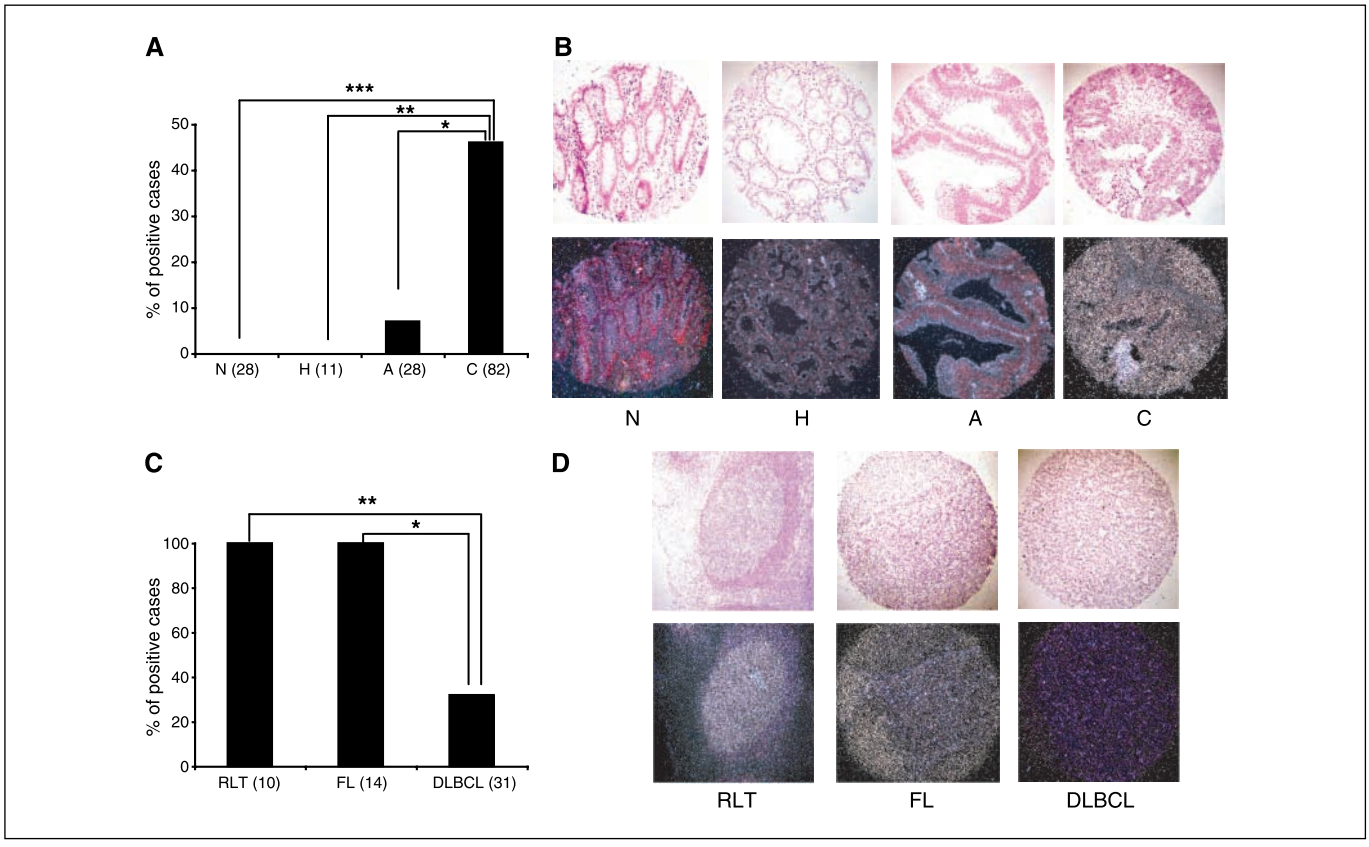

Figure 5. Expression of TP53RK in colorectal cancer progression and of STK17B in non-Hodgkin's lymphoma. *A,* TP53RK in colorectal cancer progression. Expression was evaluated by *in situ* hybridization-tissue microarray on the indicated samples (*N,* normal epithelium; *H,* hyperplastic polyp; *A,* adenoma; *C,* adenocarcinoma). The number of analyzed samples is inside the parentheses. Data are expressed as percentage of positive cases. A significant up-regulation of TP53RK was observed in adenocarcinoma compared with normal epithelium, hyperplastic polyp, and adenoma (***, $P < 0.0001$, adenocarcinoma versus normal epithelium; **, $P = 0.002$, adenocarcinoma versus hyperplastic polyp; *, $P = 0.0002$, adenocarcinoma versus adenoma). *B,* selected examples of the data shown in (*A*). In the example, normal epithelium, adenoma, and adenocarcinoma are from the same patient. *C,* STK17B in non-Hodgkin's lymphoma. Expression was evaluated by *in situ* hybridization-tissue microarray on the indicated samples (*RLT,* nonneoplastic RLT; *FL,* non-Hodgkin's lymphoma follicular type; *DLBC,* non-Hodgkin's lymphoma DLBCL. The number of analyzed samples is inside the parentheses. Data are expressed as percentage of positive samples. A significant down-regulation of STK17B was observed in DLBCL relative to RLT and follicular type (**, $P = 0.0002$, DLBC versus RLT; *, $P < 0.0001$, DLBC versus follicular type). *D,* selected examples of the data shown in (*C*). *B* and *D,* bright (*top*) and dark (*bottom*) fields are as in Fig. 3.

Table 1. Function of misregulated kinases

Kinase*	Class †	Tumor ‡	Function§	Refs.‖
AURKA	U-cI	Co	Mitosis	(21, 22)
AURKB	U-cI	La	Mitosis	(21, 22)
EIF2AK1	U-cI	Br, Co, Lu, Pr, La	Protein synthesis	(23)
LIMK1	U-cI	La, Ly	Actin dynamics	(24–26)
NEK6	U-cI	Br, Co, Lu, Ly	Mitosis	(27, 28)
NEK7	U-cI	Br, Co, La	Mitosis	(28)
RPS6KC1	U-cI	Br, Pr	Signal transduction (sphingosine-1-phosphate pathway)	(29)
SGKL	U-cI	Br	Signal transduction (phosphatidylinositol 3-kinase pathway)	(30, 31)
STK35	U-cI	Co	Actin dynamics	(32)
TP53RK	U-cI	Co	Apoptosis	(19)
CLK2	U-cII	La	Splicing	(33)
CLK4	U-cII	Co	Splicing	(34)
CRK7	U-cII	Br	Splicing	(16)
HIPK2	U-cII	Lu, Pr	Apoptosis	(35, 36)
MAP4K4	U-cII	Co, Lu, Ly	Signal transduction (MAPK pathway)	(37–39)
MINK1	U-cII	Co	Actin dynamics	(40)
PLK1	U-cII	Lu, Pr	Mitosis	(21, 41)
TAOK1	U-cII	Br, Co, Lu, La	Signal transduction (MAPK pathway)	(42, 43)
SGK2	D	Co	Signal transduction (phosphatidylinositol 3-kinase pathway)	(30, 31)
STK17A	D	Ly	Apoptosis	(20)
STK17B	D	Ly	Apoptosis	(20)

*Only kinases misregulated in at least one type of tumor in a statistically significant manner are listed.
†U-cI, up-regulated, class I; U-cII, up-regulated, class II; D, down-regulated (as from descriptions in the text).
‡Tumor types in which the indicated kinase was misregulated in a statistically significant manner: Br, breast; Co, colon-rectum, Lu, lung; Pr, prostate; La, larynx; Ly, non-Hodgkin's lymphoma.
§Function (established or putative) was obtained from the detailed descriptions presented in Supplementary Table S10. Summary functions and Gene Ontology annotations for each protein kinase were derived by merging the information obtained from the Database for Annotation, Visualization and Integrated Discovery (http://apps1.niaid.nih.gov/david/), from the GeneCards Database (http://bioinfo1.weizmann.ac.il/genecards/index.shtml), and from the SOURCE Database (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch).
‖Representative references for the function, and the involvement in cancer, of the listed kinases.

in signal transduction, actin remodeling, and control of mitotic machinery. It is of note that we also detected, in three cases, clear-cut underexpression, which suggests a putative tumor suppressor role of some kinases (see also below).

The in-depth analysis of three misregulated kinases allows further conclusions and speculations both from the point of view of the molecular understanding of cancer and from that of possible clinical applications.

First, overexpression of CRK7 correlated with indicators of aggressive disease in breast cancer, projecting a possible use of CRK7 as a prognostic marker and possibly as a therapeutic target in this malignancy. CRK7 is a protein putatively involved in the regulation of the splicing machinery (16). The occurrence of splicing defects seems to be a frequent feature of tumor cells and might contribute to malignant transformation with a variety of mechanisms (reviewed in refs. 17, 18). It is interesting that other splicing-related kinases (CLK4 and CLK2) were also found overexpressed in our screening. The molecular mechanisms responsible for splicing defects in cancer are poorly understood (17, 18). Our results raise the possibility that the alteration of regulatory kinases might be mechanistically involved in the generation of aberrant splicing patterns in cancer.

Second, in colorectal cancer, the in-depth analysis of TP53RK revealed strong correlation between its overexpression and disease progression. TP53RK is suspected to play a role in apoptosis through its ability to phosphorylate/activate p53 (19). Why a putatively proapoptotic protein should be overexpressed in cancer is not clear. However, this situation is shared with at least another proapoptotic kinase, HIPK2, which was also found overexpressed in our screening. One obvious possibility is that, similarly to what has been described for other tumor suppressor genes, such as p53, overexpression mirrors mutational inactivation (as an abortive compensatory mechanism). Thus, mutational analysis of TP53RK and HIPK2 in cancer seems an issue worthy of further investigations. Whatever the case, the strong correlation of TP53RK with disease progression in colorectal cancer suggests that it may serve as a marker for early detection of malignant transition.

Third, in STK17B, we could show a very good inverse correlation between the levels of expression of the kinase and the non-Hodgkin's lymphoma histotype, which in turn is a strong indicator of aggressive clinical behavior. STK17B (and the related kinase STK17A, which was also underexpressed in non-Hodgkin's lymphoma) is a member of the DAP subfamily, for which a proapoptotic function is well established (20). Thus, the results of our in-depth analysis reinforce the notion that some STK might play a tumor suppressor role and that their underexpression might have a causal role in certain malignancies.

One final general question concerns how our data compare with previously published high-throughput studies of alterations of STK in cancer. We did a comparison of our results with (*a*)

data extracted from ONCOMINE 3,[8] a bioinformatics platform that provides a compendium of microarray data (Supplementary Table S8), and (*b*) data extracted from several cancer kinome sequencing efforts (Supplementary Table S9). The comparison between our *in situ* hybridization-tissue microarray data and ONCOMINE revealed only partial concordance (Supplementary Table S8). This, however, is not surprising, because the two methodologies have different advantages and limitations. On the one hand, the higher sensitivity of expression profile methodologies might allow the detection of events that escape analysis by *in situ* hybridization-tissue microarray. On the other, tissue heterogeneity, which is not a problem in *in situ* hybridization-tissue microarray, may yield false positives or false negatives in expression profile analyses. Thus, the two methods should be regarded as complementary.

In the comparison between *in situ* hybridization-tissue microarray data and the cancer kinome sequencing data, we could not evidence any significant correlation (Supplementary Table S9). Thus, at least based on data available thus far, overexpression or underexpression of STK does not seem to accompany frequently their mutation.

The specific inhibition of protein kinases has emerged, in the last few years, as one of the most promising strategies for the molecular therapy of cancer. Our present study supports the idea that, in addition to tyrosine kinases, inhibiting STK might prove effective in a sizable fraction of human cancers. At the same time, our results caution about possible side effects of kinase inhibition, in the whole organism or in specific tissue/cell compartments, for those cases in which expression in normal tissues was also detected (class II kinases). It is of note that we identified a group of 10 kinases, which, although overexpressed in cancer, displayed low or absent expression in normal tissues (class I kinases). This feature makes this latter group of genes favorite candidates for diagnostic, prognostic, and therapeutic applications.

[8] http://www.oncomine.org.

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