

High-Throughput Analysis of Genome-Wide Receptor Tyrosine Kinase Expression in Human Cancers Identifies Potential Novel Drug Targets

Carsten Müller-Tidow,¹ Joachim Schwäble,¹
Björn Steffen,¹ Nicola Tidow,⁴
Burkhardt Brandt,⁴ Kerstin Becker,¹
Eric Schulze-Bahr,² Hartmut Halfter,³ Ulf Vogt,⁶
Ralf Metzger,⁵ Paul M. Schneider,⁵
Thomas Büchner,¹ Christian Brandts,¹
Wolfgang E. Berdel,¹ and Hubert Serve¹

Departments of ¹Medicine, Hematology, and Oncology, ²Cardiology, and ³Neurology and ⁴Institute of Clinical Chemistry and Laboratory Medicine, University of Münster, Münster, Germany; ⁵Department of Abdominal and Vascular Surgery, University of Cologne, Cologne, Germany; and ⁶European Laboratory Association, Ibbenbüren, Germany

ABSTRACT

Novel high-throughput analyses in molecular biology allow sensitive and rapid identification of disease-related genes and drug targets. We have used quantitative real-time reverse transcription-PCR reactions ($n = 23,000$) to analyze expression of all human receptor tyrosine kinases ($n = 56$) in malignant tumors ($n = 313$) of different origins and normal control samples ($n = 58$). The different tumor types expressed very different numbers of receptor tyrosine kinases: whereas brain tumors and testicular cancer expressed 50 receptor tyrosine kinases, acute myeloid leukemia (AML) samples expressed only 20 different ones. Specimens of similar tumor origin exhibited characteristic receptor tyrosine kinase expression patterns and were grouped together in hierarchical cluster analyses. When we focused on specific tumor entities, receptor tyrosine kinases were identified that were disease and/or stage specific. Leukemic blasts from AML bone marrow samples differed significantly in receptor tyrosine kinase expression compared with normal bone marrow and purified CD34+ cells. Among the differentially expressed receptor tyrosine kinases, we found FLT3, c-kit,

CSF1 receptor, EPHB6, leukocyte tyrosine kinase, and ptk7 to be highly overexpressed in AML samples. Whereas expression changes of some of these were associated with altered differentiation patterns (*e.g.*, CSF1 receptor), others, such as FLT3, were genuinely overexpressed in leukemic blasts. These data and the associated database (<http://medweb.uni-muenster.de/institute/meda/research/>) provide a comprehensive view of receptor tyrosine kinase expression in human cancer. This information can assist in the definition of novel drug targets.

INTRODUCTION

Receptor tyrosine kinases are a family of proteins characterized by a transmembrane domain and a tyrosine kinase motif (1). They function in cell signaling and transduce signals regulating growth, differentiation, adhesion, migration, and apoptosis (2). A wide variety of diseases, including cancer, have been linked to tyrosine kinase alterations. Indeed, at least 18 tyrosine kinases have been identified as oncogenes either in acutely transforming retroviruses or in human cancer (3). Whereas the human genome contains information about 1000 protein kinases, less than 100 tyrosine kinases have been identified. About two-thirds of these are membrane-bound receptor tyrosine kinases. Aberrant receptor tyrosine kinase activity was initially described in various epithelial cancers. Nowadays, it is well known that receptor tyrosine kinases play an important role in almost all types of cancer (4, 5).

Oncogenic activation of receptor tyrosine kinases involves point mutations and deletions as well as overexpression. Overexpression of oncogenic receptor tyrosine kinases occurs by gene amplification mechanisms (*e.g.*, epidermal growth factor receptor) and frequently by alterations of transcriptional and posttranscriptional regulatory mechanisms (6–8). The mutational activation and/or overexpression of receptor tyrosine kinases transforms cells and often plays a crucial role in the development of cancers. In addition, receptor tyrosine kinases have been shown to be involved in the development of metastatic potential as well as in response to classical therapeutics such as response to hormonal therapy for breast cancer (9).

The identification of receptor tyrosine kinases as promising targets for directed cancer therapy approaches has further increased the scientific interest in this important protein family. Humanized monoclonal antibodies have been developed against members of the epidermal growth factor receptor family, and anti-ERBB2 antibodies show significant clinical activity in breast cancer and possibly in non-small cell lung cancer (10). Similarly, pharmaceutical companies have started to develop small molecule inhibitors for receptor tyrosine kinases, and several of these have entered clinical trials and await United States Food and Drug Administration approval (11). Thus far, drug development has concentrated on a few receptor tyrosine

Received 6/19/03; revised 10/3/03; accepted 10/20/03.

Grant support: IZKF Grant (Interdisciplinary Center for Clinical Studies at the University of Münster, Department of Medicine), to the University of Münster; Deutsche Forschungsgemeinschaft Grants Mu 1328/2-3, Se 600/2-4, and SFB293-A15; and Deutsche Krebshilfe Grant 10-1539-Mü2.

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Requests for reprints: Carsten Müller-Tidow, Department of Medicine, Hematology, and Oncology, University of Münster, Domagkstrasse 3, 48129 Münster, Germany. Phone: 49-251-835-2995; Fax: 49-251-835-2673; E-mail: muellerc@uni-muenster.de.

kinases only, and drug activity is evaluated only in a subset of tumor types. One of the reasons for this limited approach is the incomplete knowledge of receptor tyrosine kinase function in many types of cancers. Whereas a few receptor tyrosine kinase families have been studied intensely in the past 20 years, the expression patterns and functions of others remains largely undiscovered. However, this information could lead to important new therapeutic approaches for presently untreatable cancers. One example is imatinib, a kinase inhibitor that blocks the function of bcr-abl but also of c-kit. Imatinib was initially prescribed in chronic myelogenous leukemia only (12). The receptor tyrosine kinase c-kit is mutated and activated in gastric stroma tumors, and treatment of these tumors with imatinib led to impressive clinical response rates (13). Thus, more detailed information about receptor tyrosine kinase expression and mutation patterns can open new fields for therapeutic approaches. Expression of several receptor tyrosine kinases has been analyzed previously in many types of cancers (reviewed in Ref. 1). However, the differences in detection procedures and the limited number of cases led only to an incomplete view of receptor tyrosine kinase expression in human cancer. Most of these studies also did not allow comparison of expression levels between different types of cancers and normal tissues. In addition, several receptor tyrosine kinases have not been studied at all for their expression patterns in cancer.

In our study we used a sensitive and quantitative reverse transcription-PCR (RT-PCR) analysis method to quantify mRNA expression of all human receptor tyrosine kinases in a wide variety of human cancers as well as in normal control tissue. Our study provides, for the first time, comprehensive expression data for all human receptor tyrosine kinases, and data analyses revealed several unexpected novel findings regarding receptor tyrosine kinase expression patterns in human cancers.

MATERIALS AND METHODS

Patients and Specimens. This study included tumor specimens and normal tissues from 372 patients with different types of cancer or control samples, all of which were obtained at the time of initial surgery or before initiation of chemotherapy. The non-small cell lung cancer patient population has been described previously (14). For leukemia samples, blasts were enriched by density centrifugation. Samples were snap frozen in liquid nitrogen and stored at -80°C . The tissue origin of the tumor samples is indicated in Fig. 1. The cDNA of various normal organs ($n = 23$) was commercially obtained [Clontech (Heidelberg, Germany) and Origene].

RNA Isolation and cDNA Preparation. The tumor samples were analyzed for the percentage of tumor cells by histology or May Grünwald Giemsa staining, and only tumor biopsies with at least 70% cancer cells were used for subsequent analyses. Similarly, cancer-free control samples were confirmed by histological examination. For total RNA preparation, samples were disrupted into small pieces, and RNA was isolated using TRIzol reagent (Life Technologies, Inc.). A total of 1 μg of RNA from each sample was reverse-transcribed using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase according to the protocol of the manufacturer

(Clontech, Palo Alto, CA). The cDNA was diluted to a total volume of 200 μl .

Primer and Probe Design. Sequence information was obtained from GenBank and previously published data (1). Primer and probes were designed using a standardized procedure: first, cDNA sequences for full-length clones were blasted against human genomic sequences to identify exon-exon junctions. Probes were designed to cross an exon-exon junction wherever possible. Sequences for primer and probe were chosen to be outside of the conserved kinase domain and outside of mutational hot spots. Primer and probes were designed using Primer Express software (Applied Biosystems). The resulting primer and probe sequences were verified in BLAST (National Center for Biotechnology Information). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe was labeled with 5'-VIC and 5'-TAMRA (15). All other probes were labeled at the 5' end with FAM and at the 3' end with the quencher TAMRA (Eurogentec). Each probe was analyzed using matrix-assisted desorption ionization-time of flight, and the reliability of PCR amplification and detection was verified on serial dilutions of standard cDNAs before analyses of patient samples. When genomic DNA was used as a template, no bands were detected after PCR amplification.

Semiautomated Quantitative RT-PCR Setup. A semi-automated PCR setup was established and used for reliable and rapid RT-PCR analysis. After reverse transcription, cDNAs were pipetted into 96-well plates. A mastermix sufficient for 412 reactions was prepared (see below), and a Tecan Genesis RP150 automated pipetting system set up the PCR reactions in 384-well plates. The PCR reaction mixture contained 600 nM of each primer and 200 nM probe in a final volume of 22.5 μl . These were PCR amplified and analyzed simultaneously in a real-time PCR machine (HT7900) that allowed analysis of 384 samples. PCR conditions were 50°C for 10 s and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Analyses of Gene Expression by Real-Time Quantitative RT-PCR. Expression levels of receptor tyrosine kinases or the housekeeping gene *GAPDH* were quantified using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System, TaqMan; Perkin-Elmer Applied Biosystems, Foster City, CA) described previously (16, 17).

Initial template concentration could be calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction. Gene expression levels were calculated as described previously (14). In brief, relative gene expression levels were calculated using standard curves generated by serial dilutions of a mixture of cDNAs representing a wide variety of normal and tumor samples. The relative expression amounts of the samples were calculated with regard to the expression levels determined in the standard curve. The SDS 2.0 software (Perkin-Elmer Applied Biosystems) was used for this purpose. Expression levels of the housekeeping gene *GAPDH* were quantitated in the same way. Expression levels for each gene and each sample were divided by the *GAPDH* expression level and used for further analysis. Several genes were independently analyzed two times, and reproducibility was always excellent, with a correlation coefficient $r > 0.95$ and $P < 0.001$.

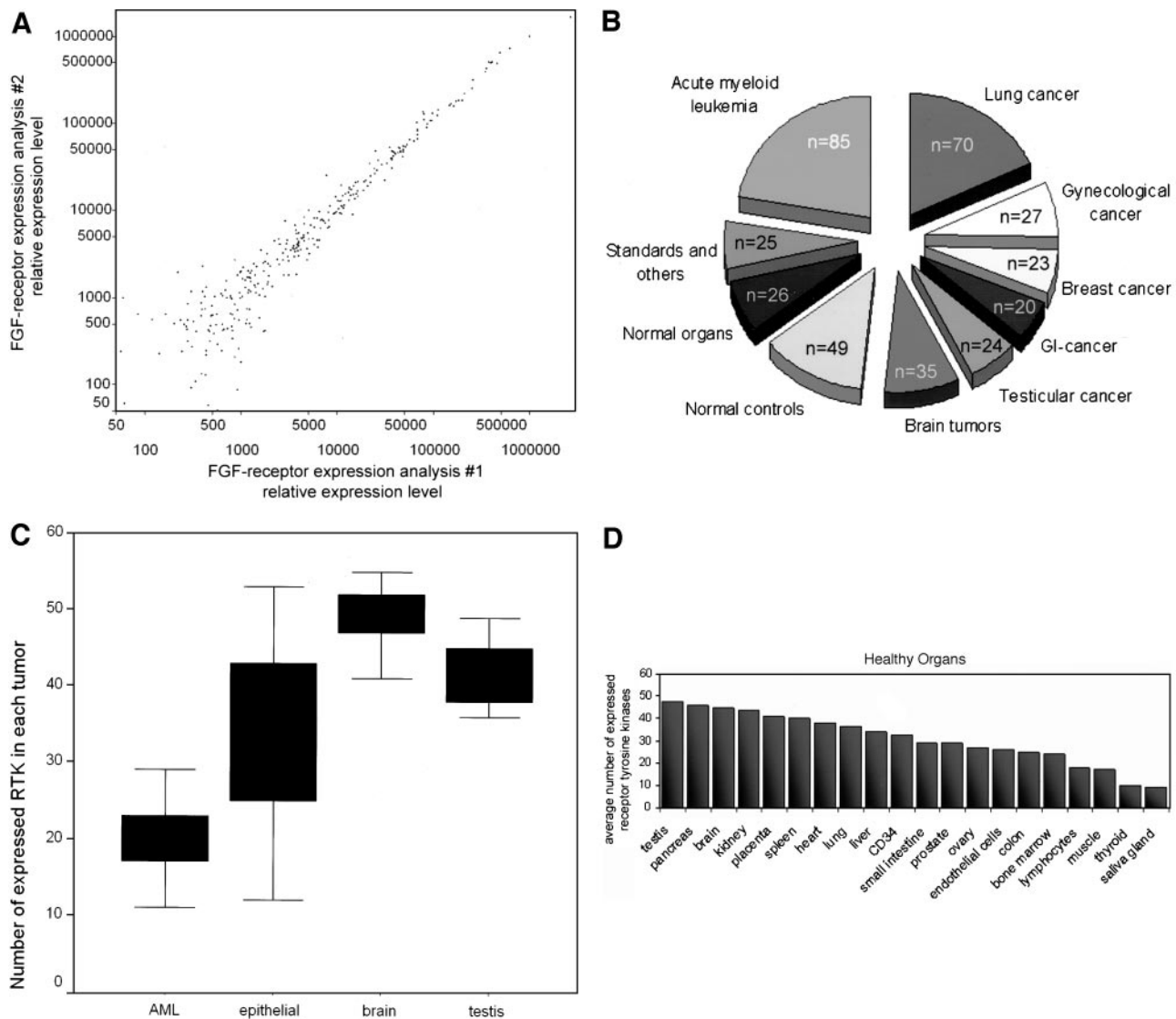


Fig. 1 Expression of receptor tyrosine kinases in human cancer as analyzed by quantitative real-time RT-PCR. **A)** This figure depicts the high degree of correlation obtained for replicate analyses of receptor tyrosine kinase expression. The example shows expression analysis of the fibroblast growth factor receptor 1. **B)** The 384 simultaneously analyzed samples included multiple tissues and tumor types as well as normal organs (pooled cDNA from several sources), control samples (control cDNA samples from single persons), and standard curves. **C)** For further analyses, lung cancer, gastrointestinal cancer, breast cancer, and gynecological cancers (ovarian, endometrial, and cervical cancer) were combined as epithelial cancers. The *box plots* indicate the numbers of receptor tyrosine kinases expressed in individual tumors. The *horizontal line within the boxes* indicates the median of expression. **D)** Expression of receptor tyrosine kinases in healthy organs. The number of expressed receptor tyrosine kinases is indicated for each analyzed healthy organ.

Analysis of FLT3 Internal Tandem Duplication (ITD) Mutations. The juxtamembrane domain of the FLT3 receptor cDNAs were amplified by RT-PCR, and one of the primers was FAM labeled. Primer sequences were 5'-GCAATTTAGGTAT-GAAAGCCAGC and 5'-CTTTCAGCATTTTGACGGCA-ACC. The wild-type receptor led to a PCR product of 240 bp that was detected by Genescan analysis (ABI3700). FLT3 ITD mutations were identified by the increased size of the PCR product. Heterozygous mutations were identified by the presence of both PCR products.

Bioinformatics and Statistics. Statistical data analyses were performed using SPSS 10.0 for Windows. The Mann-Whitney *U* test was used to compare differences between two groups, and expression levels of several groups were compared with the Kruskal-Wallis test. Survival times from acute myeloid leukemia (AML) patients were analyzed using Kaplan-Meier plots and tested for significance with the log-rank test. The Cox regression analysis for AML samples included complex karyotype, lactate dehydrogenase levels, FLT3 ITDs, and high c-kit and high ret proto-oncogene

levels. All *P*s indicate two-sided comparisons, and the level of significance was set to $P < 0.05$.

RESULTS

Analysis of Receptor Tyrosine Kinase Expression by Quantitative Real-Time RT-PCR. Based on the sequence data derived by the human genome project, a total of 58 receptor tyrosine kinase-encoding genes can be identified in humans (1). In the current study, we quantitatively analyzed expression levels of 56 human receptor tyrosine kinases in human cancers. Primers and probes labeled with FAM and TAMRA were designed for each known human receptor tyrosine kinase using a standardized procedure (see Supplementary Table 1 online at our website).⁷ With the exception of two genomic receptor tyrosine kinase sequences, which have no corresponding cDNA and where no suitable expressed sequence tag could be found in European Molecular Biology Laboratory and National Center for Biotechnology Information databases, TaqMan amplicons were successfully generated for all human receptor tyrosine kinases ($n = 56/58$). Tumor samples were generated by reverse transcription from total RNA derived from 313 tumor samples and 49 normal controls (Fig. 1). Quantitative RT-PCR reactions were performed with an automated PCR setup and a high-throughput quantitative real-time PCR machine (Applied Biosystems, HT7900) that allowed simultaneous PCR and analysis of 384 reactions. Relative receptor tyrosine kinase expression levels were calculated using standard curves derived from serial dilutions of mixed cDNA. The mixed cDNA was generated using reverse transcriptase reactions of multiple cell lines and normal tissues as well as primary tumor specimens to ensure that all human receptor tyrosine kinases were detectable in the standard curves. The expression levels of individual samples were standardized for cDNA quantity using GAPDH expression levels. The quantitative RT-PCR expression results were highly reproducible, indicating the accuracy of the method (Fig. 1A). The entire data set and the list of primers and probes used are available on our website.⁷

Tumor Types Differ in the Number of Expressed Receptor Tyrosine Kinases. Little is known about the numbers of receptor tyrosine kinases expressed in different organs and tumors. Nonetheless, this information is important to determine the presence of these potential drug targets in each type of cancer. Therefore, we initially focused on qualitative differences between receptor tyrosine kinase expression among different tumors. Samples were regarded as positive or negative based on the absence or presence of the transcript by real-time RT-PCR after 40 PCR cycles. Interestingly, the number of receptor tyrosine kinases expressed in individual tumor samples differed widely among different tumor types. On the other hand, within a given tumor histology, the number as well as the types of receptor tyrosine kinases expressed varied only to a minor degree. AML bone marrow specimens expressed, on average, 20 different receptor tyrosine kinases, whereas each brain tumor expressed about 50 different receptor tyrosine kinases (Fig. 1C).

Thus, different types of cancer exhibit strong differences in the numbers of expressed receptor tyrosine kinases. These differences in the tumors' expression patterns resembled in many cases the expression pattern in the tissue of origin. For example, the number of expressed receptor tyrosine kinases was highest in brain and testis cancer samples. Accordingly, normal testis as well as normal brain showed expression of a high number of receptor tyrosine kinases (Fig. 1D).

To identify patterns of gene expression, we used hierarchical cluster analysis. (Fig. 2). Interestingly, AML samples as well as the brain tumors clustered together, indicating the similarity of the receptor tyrosine kinase expression pattern. On the other hand, the various epithelial cancers (*e.g.*, breast, ovarian, endometrium, stomach, colon, and so forth) and the testicular cancer samples did not cluster according to their tissue of origin. These analyses indicated that certain patterns of receptor tyrosine kinase expression were closely associated with specific tumor origin.

Receptor Tyrosine Kinase Expression in AML. We focused on receptor tyrosine kinase expression in AML, a disease that holds promise for receptor tyrosine kinase inhibition (18). AML samples expressed a relatively low number of receptor tyrosine kinases (Fig. 1C), but several of the expressed receptor tyrosine kinases are well known to be crucial for hematopoiesis. Also, several receptor tyrosine kinases play an important role in leukemogenesis (19). Compared with normal control samples consisting of bone marrow samples as well as purified CD34+ hematopoietic progenitor cells, several receptor tyrosine kinases were differentially expressed in AML blast cells. Some of these expression differences could be associated, at least in part, with differentiation. For example, expression of *ptk7* was higher in CD34+ hematopoietic progenitors and in AML samples compared with the bone marrow samples that contained much higher numbers of differentiated cells (Fig. 3). However, many of the differences could not be attributed to differentiation status alone and are likely to depend on mechanisms intrinsically related to leukemogenesis. A striking result was the high level of FLT3 overexpression in AML blasts (Fig. 3). The median of FLT3 expression was almost 20-fold higher in AML blasts compared with CD34+ cells and bone marrow samples ($P < 0.0001$). To analyze a potential association between FLT3 expression and oncogenic receptor mutations, we evaluated the presence of the ITD mutations in the AML patient samples. Overall, 20.3% of the AML patients expressed a mutant FLT3 ITD allele, but few patients showed deletion of the wild-type allele. Patient samples harboring these mutations did not differ in FLT3 expression levels compared with patients expressing only wild-type FLT3 (data not shown). This indicated that FLT3 overexpression in AML patients was not related to the presence of FLT3 ITD mutations. In addition to the increased FLT3 expression, AML blasts expressed significantly higher levels of *c-kit*, *csf1* receptor, *EPHB6*, leukocyte tyrosine kinase, and *ptk7* (Fig. 3). On the other hand, expression of several other receptor tyrosine kinases was significantly reduced in AML samples compared with CD34+ and bone marrow samples derived from healthy donors. The French-American-British classification is based on the differentiation status of AML blast cells. We analyzed differentiation-associated expression differences by comparing expression in myeloblastic dif-

⁷ Supplementary data including the entire data set are available on our website: <http://medweb.uni-muenster.de/institute/meda/research/>.

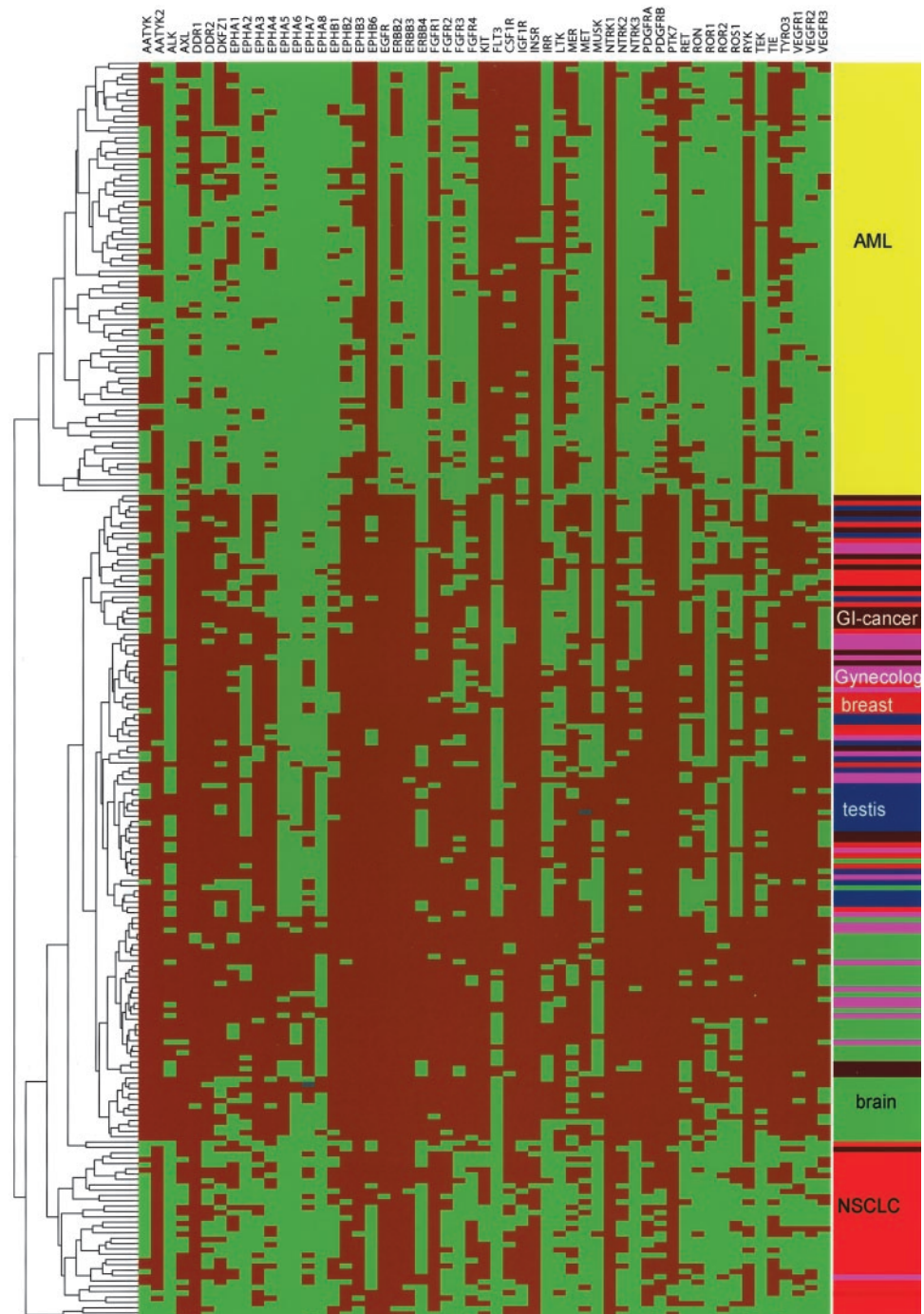


Fig. 2 Expression of receptor tyrosine kinase clusters according to tumor types. Tumors were divided into those expressing (*red*) or not expressing (*green*) a given tyrosine kinase. The binary data set was analyzed using hierarchical complete linkage cluster analysis and uncentered correlations. The results were visualized using Treeview software (35). Each tumor type is indicated by a specific color.

differentiated AML samples and in monoblastic differentiated AML samples (Fig. 3B). These analyses showed that several receptor tyrosine kinases were expressed differentially between myeloblastic and monoblastic leukemias.

Receptor Tyrosine Kinase Expression in AML Is Associated with Patient Prognosis. Recently, the prognostic impact of FLT3 tandem duplications has been demonstrated in AML patients (20, 21). To our knowledge, no other receptor

tyrosine kinase has been clearly demonstrated in AML to be associated with patient survival. In this study, the cutoff value for increased receptor tyrosine kinase expression was set at the 80th percentile of the control samples (CD34+ progenitor cells and normal bone marrow samples). Survival data were available for 63 AML patients. Expression levels of several receptor tyrosine kinases were associated with patients' relapse-free survival. Patients with high levels of c-kit (>80th percentile control

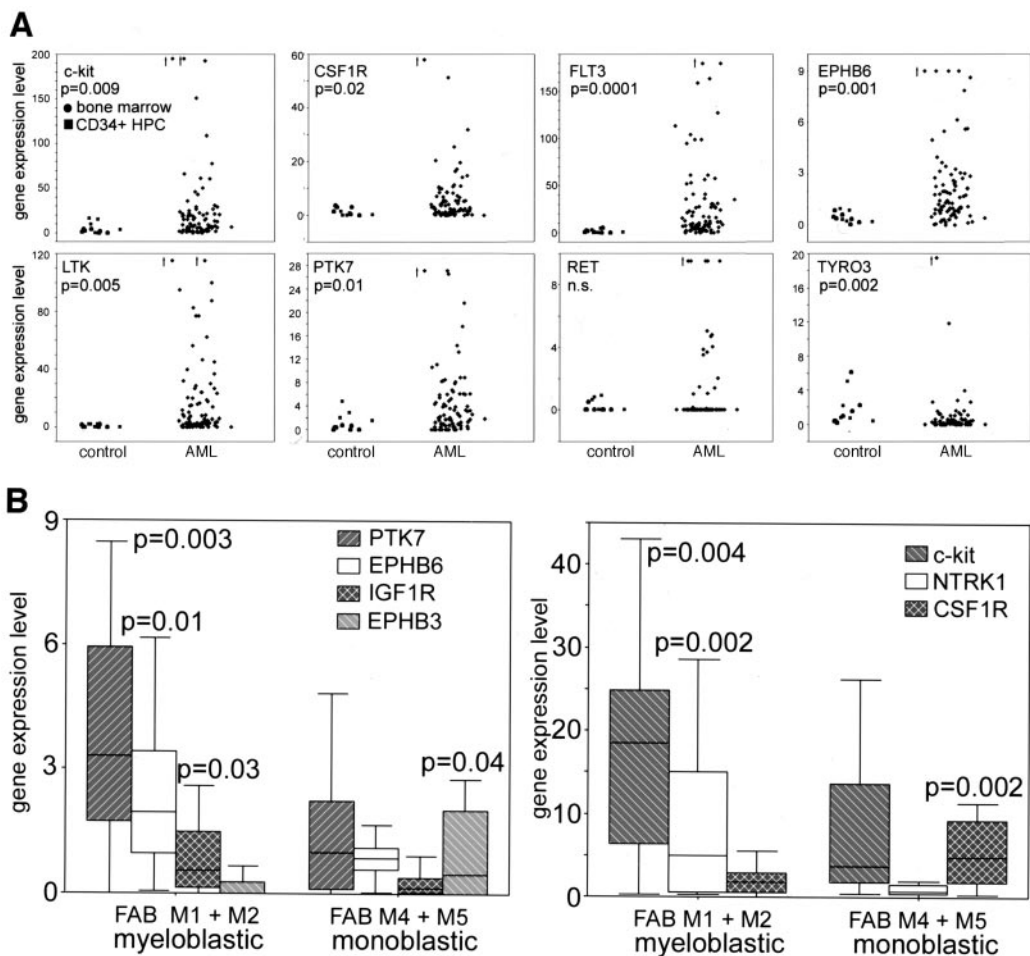


Fig. 3 Differences of receptor tyrosine kinase expression levels in acute myeloid leukemia. A) These dot plots depict the receptor tyrosine kinases with distinct expression levels between AML blasts, CD34+ progenitor cells, and normal bone marrow. Significance levels are indicated. AML blast cells usually show differentiation toward the myeloid and/or the monocytic lineage. B) We analyzed which receptor tyrosine kinases were differentially expressed in blast cells with myeloblastic morphology (French-American-British classification M1 and M2) compared with those with rather monoblastic morphology (French-American-British classification M4 and M5). The level of statistical significance of the difference in expression is indicated for each receptor tyrosine kinase (Mann-Whitney *U* test). Because different scaling was necessary, the data are presented in two panels.

samples) showed significantly better relapse-free survival and overall survival compared with patients with low levels of c-kit expression. Patients whose blasts expressed high c-kit levels experienced a mean relapse-free survival time of 543 days compared with 264 days for patients with low levels of c-kit expression ($P = 0.04$, log-rank test). For overall patient survival, differences were also significant: 235 versus 603 days ($P = 0.001$; Fig. 4). The c-kit receptor tyrosine kinase was the

only receptor that was associated with better patient survival on high expression. On the other hand, high expression levels of several other receptor tyrosine kinases were associated with shortened relapse-free survival: patients with high levels of axl kinase expression showed relapse-free mean survival times of 196 days compared with 485 days for patients with low-level axl expression ($P = 0.02$). Similar results were obtained for the csf1 receptor (262 versus 580 days; $P = 0.01$). Also, high expression

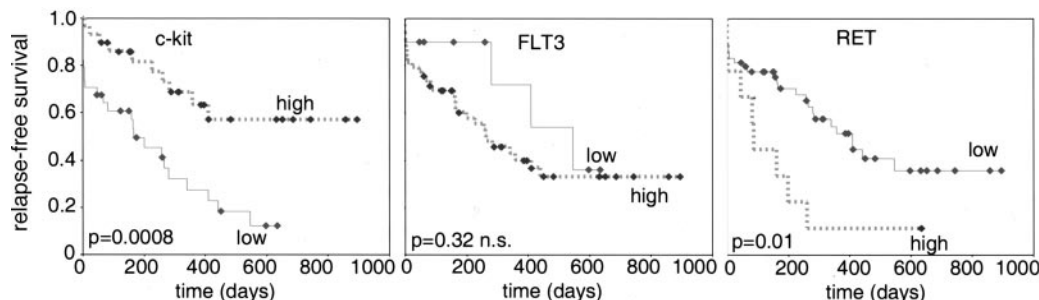


Fig. 4 Receptor tyrosine kinase expression and survival in AML. AML patients were grouped for each receptor tyrosine kinase into those with high expression (above median) and low expression (below median). Kaplan-Meier plots were drawn for relapse-free survival, and differences were analyzed by the log-rank test for statistical significance.

levels of the *ret* proto-oncogene predicted shortened relapse-free survival (210 versus 472 days; $P = 0.02$). Further analyses demonstrated that only high levels of *ret* expression were associated with significant differences in overall survival (165 versus 463 days; $P = 0.01$; Fig. 4). Whereas *axl* and *csf1* receptor were associated with shortened relapse-free survival, no significant effects on patients' overall survival could be demonstrated.

Next, we analyzed the independent prognostic value of high *c-kit* and *ret* expression levels in a multivariate Cox regression analysis. This analysis included the known prognostic determinants complex karyotype, lactate dehydrogenase, and FLT3 ITD mutations as well as *c-kit* and *ret*. Complex karyotype ($P = 0.049$) and high levels of *ret* expression ($P = 0.019$) emerged as independent prognostic parameters for AML patient survival in these analysis.

DISCUSSION

In this study, we provide for the first time a comprehensive overview of receptor tyrosine kinase expression in human cancer. The human genome contains coding information for a total of 58 receptor tyrosine kinases. For two potential receptor tyrosine kinases, only genomic data are available, and a thorough search of the databases including the expressed sequence tags did not indicate significant mRNA expression in any tissue. Thus, our analyses of 56 receptor tyrosine kinases most likely represent data of the entire set of expressed receptor tyrosine kinases in humans. These receptor tyrosine kinases were analyzed for expression in more than 350 primary tumor and control samples.

Our approach is unique in several ways: first, all samples were analyzed for expression of one receptor tyrosine kinase simultaneously, thus greatly reducing variations based on inter-assay variability. This method also allows comparison of receptor tyrosine kinase expression in various types of tumors. Second, real-time RT-PCR has evolved into a highly sensitive and reproducible system for quantitative mRNA expression analysis for minute amounts of tumor tissue (22, 23). The obtained data set provides an accurate view of receptor tyrosine kinase mRNA expression in human cancers. A receptor tyrosine kinase that is detectable by quantitative RT-PCR analysis in a specific tumor is likely to be found on the protein level as well. Consequently, the mRNA expression patterns of receptor tyrosine kinases reveal interesting information about their potential as therapeutic targets in distinct tumor entities.

Several novel findings were obtained in our study. For the first time, we could evaluate the number of receptor tyrosine kinases that are expressed in normal tissues as well as in different tumor types. Whereas some tumors such as testicular and brain cancer express almost all known receptor tyrosine kinases, a relatively low number of receptor tyrosine kinases is expressed in AML. This information is likely to be useful for elucidation of novel drug targets. The number of receptor tyrosine kinases as potential drug targets is relatively low. On the other hand, the importance of each expressed receptor tyrosine kinase for leukemia cell growth and survival might be higher than in other tumor entities. Thus, targeting of a single receptor tyrosine kinase might offer a greater chance for successful

targeting compared with other tumors. Several receptor tyrosine kinases are overexpressed and could be targets for specific therapies (e.g., FLT3, EPHB6, leukocyte tyrosine kinase, or *ptk7*). Indeed, promising responses have been observed in AML patients treated with imatinib as an inhibitor of *c-kit* or with FLT3 antagonists (24, 25). Obviously, sensitivity toward chemotherapy was not related to the number of expressed receptor tyrosine kinases. Whereas one could assume that the high number of receptor tyrosine kinases expressed in glioblastomas might contribute to the high malignancy and therapy resistance, testicular cancer, which is a highly curable disease, expressed high numbers of receptor tyrosine kinases as well.

As another finding, our study revealed that the tumor type defines the expression pattern of receptor tyrosine kinases. In cluster analysis, similar tumors clustered close together, indicating the specific receptor tyrosine kinase expression pattern associated with distinct tumor types. In most cases, these expression patterns reflect the expression associated with the corresponding normal tissue. However, tumors do express higher levels of some receptor tyrosine kinases, whereas expression of others is lost. These differences between normal tissues and the tumors provide clues regarding pathogenesis and potential for drug targeting.

Our additional analyses focused on AML. In AML, a disease in which relatively few receptor tyrosine kinases are expressed, several receptor tyrosine kinases were highly overexpressed compared with normal bone marrow and CD34+ hematopoietic progenitor cells. One of these, FLT3, is a known proto-oncogene that is frequently mutated in AML (20). Its overexpression in almost all patients with AML indicates that FLT3 overexpression might be an additional leukemogenic mechanism. Several other receptor tyrosine kinases were also expressed at much higher levels in AML samples compared with control samples. For some of these, for example, the *csf1* receptor, this might reflect the enrichment of monocytic lineage cells in monoblastic leukemia, whereas this lineage is relatively infrequent in normal bone marrow and among CD34+ progenitor cells. Others, such as *c-kit* and leukocyte tyrosine kinase, might contribute to leukemia cell survival and proliferation. The overexpressed leukocyte tyrosine kinase has previously been described to be expressed in hematopoiesis and B-cell progenitors, but its expression in AML has not been studied in detail (26).

In subsequent analyses, low *c-kit* levels and high *ret* proto-oncogene levels emerged as independent parameters for shortened overall survival. A few studies that analyzed the relationship between *c-kit* expression and survival by flow cytometry did not find an association toward prolonged survival (27–29). The clear demonstration of better survival for AML patients with high *c-kit* expression in our study is likely to depend on methodological differences in *c-kit* detection. Whereas *c-kit* might be involved in AML pathogenesis, its association with improved survival might reflect biological differences. Such biological differences [e.g., the presence of specific translocations such as $t(15;17)$ or $t(8;21)$] are well known to be pathogenetically relevant but still indicate a favorable prognosis.

The prognostic impact of high levels of *ret* expression is another novel finding of our study: *ret* has previously been shown to be expressed in a high percentage of AML samples. During normal hematopoiesis, its expression was found to be relatively low in CD34+ progenitor cells and increased during

myelomonocytic maturation. However, ret expression was absent in peripheral blood (30). Ret is a known proto-oncogene, and mutations in the ret gene cause multiple endocrine neoplasia type IIA, which is associated with medullary thyroid carcinoma and pheochromocytoma (31–33). Our finding that high-level expression of the ret proto-oncogene predicts shortened survival in AML may hint at an involvement in AML pathogenesis. Thus far, it is unclear whether somatic ret mutations occur in leukemia. In a small study on 17 AML patients no ret proto-oncogene mutations were detected (34). Further analyses are necessary to determine a potential role of ret in leukemogenesis or as a target gene for therapy.

Taken together, these data represent a comprehensive view of receptor tyrosine kinase mRNA expression in human cancer. The online data set of supplemental information⁷ will be helpful for the further study of receptor tyrosine kinases as well as for the elucidation of potential drug targets for novel therapeutic approaches.

ACKNOWLEDGMENTS

We are greatly indebted to Annette Becker for preparation of the manuscript and the figures, to Silvia Klumpen for excellent technical assistance, and to members of the laboratory for primer design and helpful discussions.

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