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TITLE: Genomic and Expression Profiling of Benign and Malignant Nerve Sheath Tumors in Neurofibromatosis Patients

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### 13. ABSTRACT (Maximum 200 Words)
The goal of this project is to identify genes involved in the malignant transformation of neurofibromas to malignant peripheral nerve sheath tumors using expression profiling and array-based comparative genomic hybridization. The significance of the genes will be validated on much larger numbers of cases using antibodies and in situ hybridization probes on tissue microarrays (TMAs). Genes will be further studied in vitro experiments using cell lines from nerve sheath tumors. While the grant starting date was on May 1, 2003 authorization to work with human subjects was not obtained until April 1, 2004. Therefore this "annual report" will only describe the actual work performed in April 2004. Nevertheless the following progress has been made in the past year: 1. The number of cases of nerve sheath tumors available will be more than sufficient to perform the first aim of this study. 2. We have started to run expression profiling and gene microarrays on a number of nerve sheath tumors and since April 1, 2004 have analyzed six malignant peripheral nerve sheath tumors and five schwannomas. 3. We have gained much experience with in situ hybridization on TMAs. This experience will enormously benefit this project.

### 14. SUBJECT TERMS
Tumor development/progression, gene microarrays, expression profiling, array-based comparative genomic hybridization, tissue microarrays, immunohistochemistry

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INTRODUCTION
Malignant transformation of benign neurofibromas is a life threatening complication in patients with neurofibromatosis. The goal of this study is to identify markers that may help the clinician determine whether a malignant transformation of a benign neurofibroma is actually in progress in a patient. We will accomplish this through a large scale genome-wide expression profiling study on benign lesions and malignant lesions originating in the nerve sheath to find genes that can function as markers of this malignant transformation. In this genome-wide search we will also use comparative genomic hybridization. In this technique, the presence of gene amplifications or deletions in tumor samples is determined. Subsequently we will validate and extend these findings on tissue microarrays (TMAs) containing very large numbers of these tumors using paraffin-embedded, formalin-fixed material. These TMAs will be examined by immuno-histochemistry with existing or de novo generated antisera or by in situ hybridization for the genes of interest.

The ultimate goal is to find markers that will help us distinguish benign from malignant nerve sheath tumors and that ultimately may lead to a serological marker to follow disease progression as well. In addition it can be expected that we may find several novel potential therapeutic targets for the treatment of malignant nerve sheath tumors.

BODY
The initiation of this project was delayed by eleven months by issues regarding the authorization to work with human subject material. However, it should be stressed that once I got into direct contact with Dr. Inese Beitins, the process actually went very quickly. I wish that I had known of her existence much earlier. On February 26 I sent her a letter after having been advised by her through a telephone conversation. Subsequently we had the approval to proceed by April 1. I mention this in detail because I can imagine that these issues might occur with other grants as well and I believe that an unnecessary delay of
ten months could have been prevented had I known of Dr. Beitins' existence so that I could have contacted her sooner.

Despite the delay incurred we have been able to make significant progress. In the past year I have inventoried the number of nerve sheath tumors currently available to me and have determined that these are sufficient to perform the initial phase of the study using gene microarrays to perform expression profiling and comparative genomic hybridization. As of April 1st, 2004, we also have started to perform these studies as described in the section below. Importantly, in parallel projects performed in my laboratory we have developed an enormous amount of experience with in situ hybridization on tissue microarrays and the experience gained in this area will be extremely valuable once we identify genes of interest in the nerve sheath tumor project.

Specific aim 1: "Genome-wide search for genes in nerve sheath tumors"

Initial expression profiling experiments using 40,000 elements cDNA microarrays were performed on six malignant peripheral nerve sheath tumors, five schwannomas and six synovial sarcomas (Figure 1). Gene filtering was then performed to remove genes that were poorly measured and to remove those genes that show no significant variation across the samples. Specifically, using selection criteria for signal over-background measurement of quality of the signal intensity 34,000 genes passed the filters. The subsequent filtering that selected for genes that varied at least fourfold in at least two of the arrays removed approximately 30,000 genes. In the final filtering step we removed genes that were not well measured in at least 80% of the data. As a result of these rather stringent filtering criteria, 1,920 genes were selected for the hierarchical clustering. The gene selection as described above is just one of many gene selections that we will perform on this data set and that we will continue to perform as the actual data set continues to grow through additions of more nerve sheath tumor specimens and other sarcomas. Unsupervised hierarchical clustering was performed on the 1,920 gene data set and as shown in Figure 1 all synovial sarcomas clustered on a branch distant from the other tumors. Likewise all schwannomas clustered on a separate branch. The malignant peripheral nerve sheath tumors showed a distribution over three branches, one of which (branch two) contained a majority (four cases) of the samples while the other two cases (STT3990 and STT3994) were on separate branches of the branch that contained that contained all schwannomas. These
findings show that malignant peripheral nerve sheath tumors are a heterogeneous group of tumors and they emphasize the need for detailed comparison with histologic findings and gene array studies. In the collaboration between myself and Drs. John Goldblum, Brian Rubin and Torsten Nielsen we have sufficient surgical pathology expertise to guarantee this.

Of course this data set is still quite small and much smaller than the one that we hope to obtain in the next 1 to 1 1/2 years. Nevertheless as an example of this study we have performed a SAM analysis to determine the genes that are most significant in the separation of malignant peripheral nerve sheath tumors from the other lesions. An example of this SAM analysis is shown in Table 1 where the 112 most significant genes that determined separation from MPNST from the other tumors are shown. Interestingly for several of these genes including CTHRC1, EGFR, we have already generated in situ hybridization probes in parallel projects on fibromatosis studies. These probes will act as validation tools of gene array data using tissue microarrays.

Specific aim 2: "Validation of candidate genes"
An important development in my laboratory is that we have become very familiar with the generation and use of in situ hybridization probes on our TMAs. In situ hybridization on formalin fixed, paraffin embedded material has long been problematic. We have started using a method (published by others) that incorporates tyramide amplification of signal to perform a non-radioactive in situ hybridization on our TMAs. In the appendix I have included a preprint of a paper, currently in press in the American Journal of Pathology, where we describe a novel marker in GIST tumors and where examples of this technique can be seen. I expect that incorporation of this technique in the nerve sheath tumor project will markedly increase the number of candidate genes that we can examine on TMAs. This is because antisera production (as proposed in the original grant application) is a very long-term proposal with a turnaround of at least 4-6 months. In contrast, in situ hybridization probes can be generated within 10 days. We still expect to generate significant numbers of conventional antisera as they will allow us to study the proteins rather than the mRNA for interesting genes but we also expect that in situ hybridization will be used as a first determination for the significance of genes identified by expression profiling or comparative genomic hybridization.
KEY RESEARCH ACCOMPLISHMENTS

1 Inventory of nerve sheath tumors available at Stanford.
This has been completed and the number of cases will be
sufficient to perform the initial phase of the study.

2 Initiation of gene array studies.
See section above.

3 Development of in situ hybridization.
We have generated a lot of experience with non-radioactive in
situ hybridization on TMAs. To date we have generated 52
probes. This will tremendously speed up our ability to examine
large numbers of genes on TMAs.

REPORTABLE OUTCOMES
No reportable outcomes are available. The study has really only
has been started for the last month.

CONCLUSIONS
The data shown above forms an important proof of principle that
we can perform these high volume experiments on the available
samples. The RNA quality was excellent from the material we
have collected and we expect to be able to analyze many more
malignant peripheral nerve sheath tumors in the next year. Our
experience with in situ hybridization will allow us to look at
larger numbers of genes in more detail with a faster turnaround.

REFERENCES:
None.

APPENDICES
Figure 1.
Table 1.
Preprint The novel marker, DOG1, is expressed ubiquitously in
GI stromal tumors irrespective of KIT or PDGFRA
mutation status. American Journal of Pathology, in
press.
Curriculum vitae Matt van de Rijn.
Figure 1 - page one of two

Figure 1 DAMD-17-03-1-0297
Legend to Figure 1

Unsupervised hierarchical clustering of gene expression profiling data performed on six synovial sarcomas, six malignant peripheral nerve sheath tumors and five schwannomas. All samples were analyzed on the same print run of 40,000 element cDNA gene microarrays. Data were entered in the Stanford microarray database and gene filtering was performed as described in the text. Subsequently the data were grouped together using the Cluster program developed by Mike Eisen that tumors with similar gene expression profiles across the genes selected were grouped together and genes that showed similar expression profiles across the 17 specimens tested were grouped together. The results were depicted in a “heat map” where the intersection between a gene (in rows) and a tumor specimen (in columns) was labeled red when the gene was relatively highly expressed in that sample compared to the other samples. The intersection was labeled green when the expression was relatively low. Using this unsupervised method of analysis it is clear that all synovial sarcomas (in blue) clustered together on one branch while all schwannomas (in green) likewise cluster on a separate branch. The majority of the MPNSTs tested so far (four cases) cluster on a branch separate from all other cases while the remaining two MPNST cases cluster on separate branches of the schwannoma group. By inspecting the heat map it can be seen that, as expected, the malignant peripheral nerve sheath tumors and the synovial sarcomas share expression in a large number of genes. However there is a significant number of genes that are unique to the MPNSTs and it will be these genes that will be initially studied in more detail if they can be verified in gene microarray experiments larger numbers of MPNSTs. The SAM analysis (Table 1) highlights 112 genes that are significant in distinguishing the 6 MPNSTs from the other specimens.
TABLE 1

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Computed Quantities

| Computed Exchangeability Factor 59 | 0.945705644 |
| 59 percentile | 0.82 |
| False Significant Number (Median, 90 percentile) | (7.08756, 29.29500) |
| False Discovery Rate (Median, 90 percentile) | (0.326812, 20.71675) |
| PPIPlot | 0.7875 |

112 Positive Significant Genes

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APPENDIX 2
The novel marker, *DOGI*, is expressed ubiquitously in GI Stromal Tumors irrespective of *KIT* or *PDGFRA* mutation status

Robert B. West¹, Christopher L. Corless², Xin Chen⁷, Brian P. Rubin³, Subbaya Subramanian¹, Kelli Montgomery¹, Shirley Zhu¹, Catherine A. Ball⁴, Torsten O. Nielsen⁵, Rajiv Patel⁶, John R. Goldblum⁶, Patrick O. Brown⁷, Michael C. Heinrich⁸, Matt van de Rijn¹.

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Abstract:

We recently characterized gene expression patterns in GISTs using cDNA microarrays, and found that the gene FLJ10261 (DOG1, Discovered on GIST-1), encoding a hypothetical protein, was specifically expressed in GISTs. The immunoreactivity of a rabbit antiserum to synthetic DOG1 peptides was assessed on two soft tissue tumor microarrays (TMAs). The TMAs included 587 soft tissue tumors, with 149 GISTs, including 127 GIST cases for which the KIT and PDGFRα mutation status was known. Immunoreactivity for DOG1 was found in 136 of 139 (97.8%) of scorable GISTs. All 7 GIST cases with a PDGFRα mutation were DOG1 positive, while most of these failed to react for KIT. The immunohistochemical findings were confirmed with in situ hybridization probes for DOG1, KIT and PDGFRα. Other neoplasms in the differential diagnosis of GIST, including desmoid fibromatosis (0/17) and Schwannoma (0/3), were immunonegative for DOG1. Only 4 out of 438 non-GIST cases were immunoreactive for DOG1.

DOG1, a protein of unknown function, is expressed strongly on the cell surface of GISTs and is rarely expressed in other soft tissue tumors. Reactivity for DOG1 may aid in the diagnosis of GISTs, including PDGFRα mutants that fail to express KIT antigen, and lead to appropriate treatment with imatinib mesylate, an inhibitor of the KIT tyrosine kinase.
Introduction:

Gastrointestinal stromal tumors occur in the wall of the bowel and have been proposed to arise from the interstitial cells of Cajal. The differential diagnosis of these tumors includes desmoid fibromatosis, Schwannoma, leiomyosarcoma, and, in some cases, high grade sarcomas. Accurate diagnosis of GIST is important, because imatinib mesylate has been shown to significantly inhibit these tumors presumably through inhibition of the KIT tyrosine kinase receptor, which is highly expressed in these tumors. As a result, the diagnosis of GIST relies heavily on KIT immunoreactivity. Current recommendations in the literature emphasize a diffuse, strong KIT immunoreactivity for the diagnosis of GIST. CD34 immunostaining can also aid in the diagnosis, but a subset of cases is immunonegative while many other types of sarcomas are immunoreactive for this marker. In the vast majority of GISTs, high levels of KIT expression are accompanied by a KIT gene mutation in exon 9, 11, 13 or 17.

Recently, a subset of GISTs have been found to have \textit{PDGFRA} mutations rather than \textit{KIT} mutations. Patients with GISTs containing mutations in \textit{PDGFRA} may still benefit from imatinib therapy, but these tumors often fail to react with antibodies against KIT and hence may remain undiagnosed as GIST. In addition, some GISTs with KIT mutations may have low KIT expression by immunohistochemistry yet will still respond to imatinib therapy.

Although much work has been done on the biology of GISTs and KIT, additional insight has recently been gained through gene microarray studies. These studies have identified a number of genes whose expression is relatively increased compared to other soft tissue tumors. This includes genes known to be involved with GISTs, such as
KIT and CD34, but also includes a number of genes that have not been well characterized. We have generated an antiserum against one GIST specific gene, encoding for the hypothetical protein FLJ10261, which we have named "Discovered on GIST 1" (DOG1). Using immunohistochemistry with this antiserum and in situ hybridization with DOG1-specific probes, we show that DOG1 is highly expressed not only in typical GISTs but also in KIT-mutation negative GIST.
Materials and Methods:

Tissue Microarray

The studies described here were performed with the approval of the Institutional Review Board at Stanford University Hospital. Two TMAs were used for this study. The first TMA contained 460 different soft tissue tumors from 421 patients, with each tumor represented by two cores. The samples were distributed over two array blocks that were constructed using a technique previously described\textsuperscript{19} with a tissue arrayer from Beecher Instruments, Silver Spring, MD. 0.6 mm cores were taken from paraffin embedded soft tissue tumors archived from the Stanford University Medical Center between 1995 and 2001. This array has also been used for characterization of Apolipoprotein D expression\textsuperscript{20}. The second TMA used GISTs that were obtained from the pathology archives of Oregon Health and Science University Hospital, the Portland VA Medical Center and the Kaiser Permanente Northwest Regional Laboratory. This single-block array consisted of 0.6 mm cores from formalin-fixed, paraffin-embedded tumor assembled using a semi-automated tissue arrayer\textsuperscript{21}. There was one core for each tumor, and all of the GISTs on this TMA were analyzed for mutations in exons 9, 11, 13 and 17 of the \textit{KIT} gene using a combination of denaturing HPLC and direct sequencing, as previously described\textsuperscript{13,22}. \textit{KIT} wild-type tumors included on the array were also screened for mutations in exons 12 and 18 of the \textit{PDGFRA} gene\textsuperscript{13}.

Antibody Generation

The cDNA-derived protein sequence of \textit{DOG1} showed no significant homology with other genes, including the \textit{KIT} gene. A rabbit polyclonal antibody was raised by
injecting 3 peptides derived from the gene sequence (Applied Genomics Inc. (AGI), Hunstville, AL). These peptides have no sequence homology to KIT. The peptides were synthesized by standard FMOC chemistry: Peptide 1 EEAVKDHPRAEYEARVLEKSLK; Peptide 2 DHEECVKRKQRYEVDYNLE; Peptide 3 KEKVMLVELFMREEQDK. The peptides were conjugated to KLH and injected into two out-bred rabbits. The serum (S284) was harvested after the rabbits demonstrated a significant anti-peptide titer. Affinity-purified antibodies were obtained by passing the antiserum over an affinity column conjugated with the three peptides; bound antibodies were eluted with a pH gradient.

**Immunohistochemistry**

Primary antibodies were directed towards DOG1 (S284, AGI, Rabbit polyclonal, 1:50) and KIT (DAKO, Carpinteria, CA Rabbit polyclonal, 1:50). Serial sections of 4 μM were cut from the tissue array blocks, deparaffinized in xylene, and hydrated in a graded series of alcohol. Staining was then performed using the EnVision+ anti-rabbit system (DAKO).

**In situ hybridization**

In situ hybridization of TMA sections was performed based on a protocol published previously\(^23\)\(^24\). Briefly, digoxigenin (DIG)-labeled sense and anti-sense RNA probes are generated by PCR amplification of 400 to 600 bp products with the T7 promoter incorporated into the primers. In vitro transcription was performed with a DIG RNA-labeling kit and T7 polymerase according to the manufacturer’s protocol (Roche...
Diagnostics, Indianapolis, IN). 5um thick sections cut from the paraffin blocks, deparaffinized in xylene, were hydrated in graded concentrations of ethanol for 5 minutes each. Sections were then incubated with 1% hydrogen peroxide, followed by digestion in 10ug/ml of proteinase K at 37°C for 30 minutes. Sections were hybridized overnight at 55°C with either sense or antisense riboprobes at 200ng/ml dilution in mRNA hybridization buffer (Dako). The following day, sections were washed in 2xSSC and incubated with 1:35 dilution of RNase A cocktail (Ambion, Austin, TX) in 2xSSC for 30 minutes at 37°C. Next, sections were stringently washed in 2X SSC/50% formamide twice, followed by one wash at 0.08X SSC at 50°C. Biotin blocking reagents (Dako) were applied to the section to block the endogenous biotin. For signal amplification, a HRP-conjugated rabbit anti-DIG antibody (Dako) was used to catalyze the deposition of biotinyl tyramide, followed by secondary streptavidin complex (GenPoint kit; Dako). The final signal was developed with DAB (GenPoint kit; Dako), and the tissues were counterstained in hematoxylin for 15 seconds.

Scoring of Immunohistochemistry and in situ hybridization

Cores were scored as follows. A score of “0” was given for absent or insignificant staining: less than 5% tumor cells with light brown staining. A score of “1” was given for unscorable cores. A score of “2” was given for light brown stain in greater than 5% of tumor cells or dark brown stain in less than 50% of tumor cells. A score of “3” was given for dark brown staining in greater that 50% tumor cells. Non-tumor cells and cells of unknown origin were not scored. The cores were independently reviewed by two
pathologists (RBW and MvdR) and disagreements were reviewed together to achieve a consensus score.

*Digital image collection and data analysis*

To aid in the analysis of numerous tissue cores stained by immunohistochemistry and in situ hybridization, digital images were collected using the BLISS instrument (Bacuslabs, Lombard IL; http://bacuslabs.com). Scoring results were combined using Deconvoluter and represented in Treeview \(^{25}\), as shown on the accompanying website (http://microarray-pubs.stanford.edu/tma_portal/dog1/), where over 4,000 digital images are available.
Results:

Previously, we examined the gene expression profile of GISTs using cDNA microarrays and identified a number of the genes, in addition to the *KIT* gene, that demonstrated a specific pattern of elevated mRNA expression in GISTs\(^1\). Figure 1 shows the relative level of mRNA expression for one of these genes, *DOG1* (*FLJ10261*), compared with *KIT* in a variety of soft tissue tumors, including those in the differential diagnosis of GIST. Searches failed to show any sequence similarity between the genes on either the DNA or protein level.

A rabbit antiserum was generated against synthetic peptides derived from the putative coding sequence of DOG1. Antiserum immunoreactivity was characterized on two separate TMAs containing soft tissue tumors. The first TMA contained 460 different soft tissue tumor samples representing over 50 different diagnostic entities\(^2\). This array included 22 KIT-immunoreactive GISTs. The second TMA included 127 GIST cases for which the *KIT* and *PDGFRA* mutation status was previously determined. On this TMA there were 102 cases with an activating mutation in *KIT*, 8 cases with a mutation in *PDGFRA*, and 17 cases that were wild-type for both kinases but nevertheless had clinical, histologic, and immunophenotypic features typical for GIST.

In these two TMAs, 136 of 139 scoreable GISTs (97.8%) demonstrated immunoreactivity with DOG1 antiserum (Figures 2 and 3, Table 1). The staining observed with DOG1 antisera appeared predominately localized to the plasma membrane (Figure 4A). In some very strongly immunoreactive samples, the subcellular distribution of the staining could not be evaluated (Figure 4B). Mast cells present in some of the samples, for example synovial sarcoma, were strongly immunoreactive as well (Figure
4C), while the same samples showed only weak staining in the mast cells with KIT antibodies. We confirmed these results with in situ hybridization studies (Figures 5 and 6). Interestingly, DOG1 antisera stained all 8 scorable PDGFRA-mutant GISTs (1 case from 1st TMA and 7 cases from 2nd TMA), while the KIT antibody staining was weak in 3 of these cases and negative in the remaining 5. These findings were further extended by in situ hybridization with PDGFRA (Figure 6). PDGFRA expression was predominately, but not exclusively, present in the PDGFRA-mutant GISTs. 5 of 6 (83%) scorable PDGFRA-mutant GISTs were positive for PDGFRA ISH (Figure 2 and 3, table 1). In contrast, only 10 of 70 (14%) KIT-mutant and KIT-wildtype GISTs were positive for PDGFRA ISH. Correlation of KIT ISH with KIT immunohistochemistry was good, with the ISH signal detectable in almost all immunopositive cases (Figure 2). However, a difference was seen in the PDGFRA-mutant GISTs with regard to KIT expression. Three cases were immunopositive for KIT, but only one case was positive by KIT ISH.

Hierarchical clustering analysis of IHC and ISH data was performed as previously described. Among these parameters – KIT IHC, KIT ISH, DOG1 IHC, DOG1 ISH, and PDGFRA ISH – the most distinguishing feature was PDGFRA ISH positivity (Figure 2), with overexpression of PDGFRA by PDGFRA ISH seen in only in a small subset of GISTs. Images of all cores from both TMAs were digitally captured and are available at the accompanying website (http://microarray-pubs.stanford.edu/tma_portal/dog1/).

From the 460 tumor samples that were not classified as GIST in the first TMA, only four cases that were not histologically and immunophenotypically consistent with GIST were immunoreactive with DOG1 antiserum: 1 synovial sarcoma (1/20 = 5%), 1 (1/40 = 2.5%) leiomyosarcoma, 1 (1/4 = 25%) fibrosarcoma, and (1/9 = 11%) 1 Ewing’s
sarcoma/PNET. Of the 40 leiomyosarcomas, 17 originated in the abdomen and none of these were DOG1 immunoreactive. Other tumors in the GIST differential diagnosis failed to stain with the DOG1 antisera. These include desmoid fibromatosis (17 cases) and Schwannoma (3 cases). Parenthetically, under the staining conditions used, none of the fibromatosis cases were positive for KIT by immunohistochemistry or in situ hybridization. One leiomyosarcoma was positive for KIT immunohistochemistry only (TMA 3725). Interestingly, the staining was exclusively in a diffuse nuclear pattern. This tumor was negative for DOG1 by both immunohistochemistry and in situ hybridization and for KIT in situ hybridization.

Seven cases in the first TMA, not counted among the 22 unequivocal GISTs, showed histologic features indeterminate between GIST and smooth muscle tumor. All of these tumors were located in the wall of the stomach or intestine, with four tumors from the stomach, one from the duodenum, one from the gastro-esophageal junction, and one from the rectum. All seven cases were negative for KIT by immunohistochemistry and thus might not be considered GISTs according to current recommendations. However, four of the seven cases were positive by KIT in situ hybridization, while DOG1 immunoreactivity was seen in two cases, and all seven cases were positive for DOG1 by in situ hybridization. Furthermore, two cases (TMA 863 and 3696) were positive for PDGFRA in situ hybridization. Subsequent sequence analysis of cases 863 and 3696 revealed a point mutation and a deletion in exon 18 of PDGFRA, respectively. To date, such mutations have only been described in GISTs. We conclude that the seven KIT immunonegative cases with morphologic features between GIST and smooth muscle tumor actually represent GISTs.
We also stained a tissue microarray containing a spectrum of normal tissues with the DOG1 antiserum (data not shown). We observed staining in the epithelium of breast, prostate, salivary gland, liver, stomach, testis, pancreas, and gallbladder. The pattern of DOG1 immunostaining of the Interstitial Cells of Cajal was similar to KIT. In addition, DOG1 antiserum reacted with a number of tumor cores in a carcinoma array, including some that did not stain with KIT antiserum (data not shown).
Discussion:

GISTs have a high rate of local recurrence. Imatinib, a small molecule inhibitor of several type III receptor tyrosine kinases, including KIT and PDGFRA, has demonstrated promise in controlling GIST growth. The majority of GISTs (80-85%) harbor oncogenic mutations of KIT, and for this reason KIT has been regarded as the primary target for imatinib therapy. Indeed, initial trials of imatinib were limited to KIT-immunoreactive GISTs. Recently it was discovered that a subset of GISTs (5-7%) has activating mutations of PDGFRA. Most of these tumors are weak or negative in immunostaining for KIT, which may lead to underdiagnosis and possible withholding of imatinib therapy. Furthermore, identification of PDGFRA-mutant GISTs requires molecular analysis, a laborious process that is not ideal for application in a routine clinical setting.

In this paper, we demonstrate that a novel gene, DOG1, identified in a DNA microarray analysis of gene expression patterns as associated with GIST, is highly expressed in both KIT- and PDGFRA-mutant GISTs. Expression of DOG1 in GISTs was demonstrated both by immunodetection of the protein and by in situ hybridization. DOG1 immunoreactivity was assessed on two soft tissue tumor microarrays representing 587 soft tissue tumors, including 149 GISTs. 98.7% of scorable GISTs demonstrated immunoreactivity with DOG1 antisera. Only four KIT-negative, non-GIST soft tissue tumors were DOG1 immunoreactive. Several GISTs with mutations in the PDGRFA gene were found to react only by in situ hybridization for DOG1 and to be negative for DOG1 by immunohistochemistry. Future studies are necessary to determine whether monoclonal antibodies against purified DOG1 might yield tools with sensitivity similar to
that seen with in situ hybridization probes. We also confirm PDGFRA expression in a subset of GISTs using in situ hybridization. PDGRFA expression and KIT expression are not mutually exclusive. A subset of KIT-mutated GISTs expresses PDGRFA in addition to KIT while a subset of PDGRFA-mutated tumors also expresses KIT. These data were seen with both immunohistochemical and in situ hybridization techniques.

In addition to the marked similarity in reactivity for DOG1 protein on non-GIST sarcomas, DOG1 protein can also be seen in a subset of melanomas and germ cell tumors as has been described for KIT (West et al., in preparation). Furthermore just as seen with the KIT molecule, a variety of carcinomas also express DOG1. These tumors mostly overlap with the KIT positive tumors. While within the field of soft tissue tumors DOG1 expression appears quite specific for GIST, in a differential diagnostic setting DOG1 reactivity does not exclude carcinomas. Therefore additional markers such as keratin stains should be performed when the differential diagnosis includes carcinoma.

We also demonstrated the feasibility of assessing GIST markers by in situ hybridization on paraffin embedded tissue. Correlation between immunohistochemistry and ISH for DOG1 on GISTs was excellent. In the case of KIT, the correlation was not as strong due to relatively weak or absent ISH signals in some CD117-positive GISTs. It is likely that this reflects lower sensitivity of the KIT ISH assay, although cross-reactivity of the CD117 antibody to another epitope on GISTs has not been excluded. In situ hybridization for PDGFRA proved to be valuable in identifying KIT-negative GISTs, although DOG1 immunohistochemistry was equally sensitive for these cases. Overall, we have found that ISH techniques are complementary to IHC tests in the evaluation of GISTs.
DOGI has been recently identified as a gene in the CCNDI-EMS1 locus on human chromosome 11q13, which is amplified in esophageal cancer, bladder tumors, and breast cancer\textsuperscript{26}. Human DOGI protein showed 89.8\% total-amino-acid identity with mouse DOGI protein, and also 58.4\%, 38.3\%, and 38.6\% identity with human C12orf3, C11orf25, and FLJ34272/BAC03704 proteins, respectively. Sequence analysis predicts the presence of eight transmembrane spanning segments. This correlates with our observations of the immunohistochemical localization to the cell membrane. DOGI may be part of an as yet unclassified ion transporter family.

Since the biologic function is unknown, it is unclear why DOGI is so widely expressed in GISTs. Two broad possibilities exist. It may be that the protein has a role in receptor kinase type III signal transduction pathways. On the other hand, DOGI may be a fortuitous marker of the GIST phenotype, with no direct connection to the KIT and PDGFRA signaling pathways. The finding that mast cells are also immunoreactive for DOGI tends to favor the former possibility.

In summary, we demonstrate that detection of a novel gene, DOGI, identifies the vast majority of both KIT- and PDGFRA- mutated GISTs. This may be of clinical value in identifying candidates for Gleevec therapy. As a cell membrane associated protein, with markedly elevated expression in GISTs, DOGI may also be a potential therapeutic target.

ACKNOWLEDGEMENTS:

This work was supported by NIH grants CA85129 and CA84967 and the Howard Hughes Medical Institute. P.O.B. is an Associate Investigator of the Howard Hughes Medical...
Institute.
**Figure Legends.**

Figure 1: Gene array measurement of $KIT$ and $DOG1$ mRNA expression in 30 soft tissue tumors. Red indicates a relatively high level of expression while green denotes a low level of expression. Gene array data for STTs 524, 629, 417, 418, 219, 111, 656, 94, 335, 794, 1148, 850, 616, 710, 523, 526, 740, 607, and 1220 have been previously reported$^{18}$.

Figure 2: Hierarchical clustering of CD117 IHC, $CD117$ ISH, $PDGFR\alpha$ ISH, DOG1 IHC, and $DOG1$ ISH. The results for GISTs on the 2 TMAs have been combined. Antisera or hybridization probes are in columns, tumors in rows. Bright red denotes strong reactivity, while dark red and green indicate low and absent reactivity, respectively. White means missing data.

Figure 3: Staining results on GISTs for CD117 IHC, $CD117$ ISH, $PDGFR\alpha$ ISH, DOG1 IHC, and $DOG1$ ISH in graphic form (see also Table 1).

Figure 4: Immunohistochemical staining with anti-DOG1 serum (S284) and KIT on 2 GISTs (TMA 822 (A) and 3688 (B)) and a synovial sarcoma (TMA 856 (C)).

Figure 5: In situ hybridization of a GIST and leiomyosarcoma with antisense probes to $DOG1$ and $KIT$ on a GIST and a leiomyosarcoma (LMS). The corresponding negative control sense probes are included in the inset in the upper right hand corner of the GIST sample.
Figure 6: In situ hybridization of \textit{KIT}, \textit{DOG1}, and \textit{PDGFRA} with GISTs.

A) GIST with mutation in \textit{KIT} shows positive ISH for \textit{KIT}, \textit{DOG1} but not \textit{PDGFRA}.

B) GIST with mutation in \textit{PDGFRA} shows positive ISH for \textit{DOG1} and \textit{PDGFRA} but not for \textit{KIT}.

C) Negative control leiomyosarcoma.
Table 1: Staining results for CD117 IHC, *CD117* ISH, *PDGFRA* ISH, DOG1 IHC, and DOG1 ISH in tabular form (see also Figure 3).

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Figure 1
Figure 3

GIST markers by mutation status

% Positive

wt  KIT ex 9  KIT ex 11  KIT ex 13  KIT ex 17  PDGFRα  unknown

Mutation status

CD117  CD117 ISH  Pdgfra ISH  DOG1  DOG1 ISH
Figure 4

A) GIST TMA 822

B) GIST TMA 3688

C) SynSarc TMA 856
Figure 5

GIST    LMS

DOG1

KIT
CURRICULUM VITAE

Matt van de Rijn, M.D., Ph.D.

PERSONAL DATA

Name: Jan Matthijs van de Rijn
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State of Pennsylvania: MD 055043-L, 8/95
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EDUCATION AND APPOINTMENTS

1974-1979: Medical student at the University of Amsterdam
Received Master's degree in Medicine

1/80-1/82: Graduate student at The Netherlands Cancer Institute
Amsterdam. Dr. Jo Hilgers, adviser

1/82-6/84: Visiting graduate student at the Dana-Farber Cancer Institute
Harvard Medical School, Boston. Dr. Cox Terhorst, adviser

12/84: Received Ph.D., University of Amsterdam
Dr. Piet Borst, thesis supervisor
Field of study: Protein chemistry of T lymphocyte cell
surface antigens

1/85-10/86: Clinical rotations at the Academic Medical Hospital
University of Amsterdam

7/86: Passed ECFMG (Educational Commission for Foreign Medical
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10/86: Received M.D., University of Amsterdam

11/86-2/89: Postdoctoral fellowship in the laboratory of Dr. I. L. Weissman
Department of Pathology, Stanford University
First year funded by a grant from The Netherlands
Organization for the Advancement of Pure Research
From November 87 funded by a postdoctoral grant from the Multiple Sclerosis Society
Field of study: Protein chemistry and genetic analysis of lymphocyte homing receptors

2/89-6/91: Residency in pathology at Stanford University Medical Center

7/91-6/92: Fellowship in surgical pathology, Department of Pathology
           Stanford University Medical Center
           Co-chief resident in surgical pathology,
           Department of Pathology, Stanford University Medical Center

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7/93-6/94: Clinical Fellow, Department of Pathology
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7/94-6/95: Clinical Assistant Professor, Department of Pathology
           Stanford University Medical Center

7/95-6/98: Assistant Professor, Department of Pathology and Laboratory
           Medicine, University of Pennsylvania Medical Center

7/98-6/2001: Assistant Professor, Department of Pathology
             Stanford University Medical Center.

7/2001-present Associate Professor, Department of Pathology
                 Stanford University Medical Center.

MEMBERSHIPS
United States and Canadian Academy of Pathology
South Bay Pathology Society
American Society for Investigative Pathology
American Association for Cancer Research

Ad hoc referee for:
American Journal of Clinical Pathology
American Journal of Pathology
Blood
Medical and Pediatric Oncology
Southern Medical Journal
The Journal of Histochemistry and Cytochemistry
Cancer
Modern Pathology  
British Journal of Cancer  
Cell Biochemistry and Biophysics  
The Lancet  
New England Journal of Medicine  
Clinical Cancer Research

NIH/NCI GRANT REVIEWS, WORKING GROUPS, NATIONAL PANELS  
Reviewer for RFA: "Technologies for gene expression in the nervous system"  

Participant and presenter at NCI “Working Group Meeting on Tissue Arrays.”  
December 16-17, 1999, Rockville, MD.

Participant NCI “State of the Science Sarcoma” meeting June 17-18, 2002, Bethesda, MA

Participant and co-chair of breakout session at NCI “Sarcoma Progress Review Group Roundtable Meeting”. October 8-10, 2003, Philadelphia, PA

Member site-visit committee at Erasmus Postgraduate School of Molecular Medicine, November 12-14, 2003, Rotterdam, The Netherlands.

EDITORIAL BOARDS  
2003  PLOS: Public Library of Science  
2004  The American Journal of Pathology

COURSES/MEETINGS


2. The use of molecular biology and immunohistochemistry in the differential diagnosis of soft tissue tumors (with Fred G. Barr, M.D., Ph.D.), April 2000, American Society of Clinical Pathologists, Boston, MA.

3. Co-director and speaker at the 2002 Special Course for Advanced Molecular Pathology at the 91st annual meeting of the United States and Canadian Academy of Pathology, Chicago, IL.

4. Co-chair Prefered Papers section: Bone and Soft Tissue Tumors at the 93rd annual meeting of the United States and Canadian Academy of Pathology, Vancouver, Canada.
5. Co-director of the 2004 Special Course for Advanced Molecular Pathology at the 93rd annual meeting of the United States and Canadian Academy of Pathology, Vancouver, Canada.

INVITED LECTURES

1. EBV positive lymphomas. The Netherlands Cancer Institute, October 1994, Amsterdam, The Netherlands.


10. Chairman of session “Functional Genomics” and talk “Western blot analysis and RNA expression” at Advances in Laser Capture Microdissection, June 1999, NIH, Bethesda, MD.


16. Study of human tumors using cDNA and tissue microarrays. Vanderbilt University, November 2000, Nashville, TN.


30. Data management for tissue microarrays. October 4, 2002, John Radcliffe Hospital, Oxford University, Great Britain.


32. **Keynote Address:** The use of gene arrays and tissue microarrays for the study of human tumours. October 9, 2002, 24th International Congress of the International Academy of Pathology, Amsterdam, The Netherlands.


37. Using tissue microarrays in high throughput studies. Director's Challenge meeting, NCI. November 7, 2002, Bethesda, MA.

38. Hardware and software used in gene microarray analysis. 7th ADNAT meeting, CCMB Research Center, March 1, 2003, Hyderabad, India.


40. A demonstration of the Stanford Microarray Database. 7th ADNAT meeting, CCMB Research Center, March 6, 2003, Hyderabad, India.

41. High throughput studies on sarcomas. 7th ADNAT meeting, CCMB Research Center, March 7, 2003, Hyderabad, India.
42. Array based comparative genomic hybridization and laser capture microdissection. 7th ADNAT meeting, CCMB Research Center, March 8, 2003, Hyderabad, India.

43. Expression profiling, comparative genomic hybridization and tissue microarray studies on sarcomas. Dept of Pathology, Virginia Commonwealth University, March 21, 2003, Richmond, VA.


52. Gene and tissue microarray studies on sarcomas. October 22, 2003, Hospital Sant Pau, Barcelona, Spain.

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IN PREPARATION