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## Comprehensive genomic analysis reveals clinically relevant molecular distinctions between thymic carcinomas and thymomas

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### Abstract

**Purpose**—Thymomas and thymic carcinomas are rare intrathoracic malignancies that can be invasive and refractory to conventional treatment. Because these tumors both originate from the thymus, they are often grouped together clinically. However, whether the underlying biology of these tumors warrants such clustering is unclear, and the optimum treatment of either entity is unknown.

**Experimental design**—All thymic tumors were profiled for mutations in genes encoding components of the EGFR and KIT signaling pathways, assessed for EGFR and KIT expression by immunohistochemistry (IHC), and analyzed by array-based comparative genomic hybridization (aCGH). Previously untreated tumors were subjected to global gene expression arrays.

**Results**—We analyzed 45 thymic tumors (thymoma n=38 (type A: n=8, type B2: n=22, type B3: n=8), and thymic carcinoma n=7). One thymoma and one thymic carcinoma harbored *KRAS* mutations (G12A and G12V, respectively), and one thymoma had a G13V *HRAS* mutation. Three tumors displayed strong KIT staining. Two thymic carcinomas harbored somatic *KIT* mutations (V560del and H697Y). In cell viability assays, the V560del mutant was associated with similar sensitivities to imatinib and sunitinib, while the H697Y mutant displayed greater sensitivity to

sunitinib. Genomic profiling revealed distinct differences between type A-B2 thymomas vs. type B3 and thymic carcinomas. Moreover, aCGH could readily distinguish squamous cell carcinomas of the thymus vs. the lung, which can often present a diagnostic challenge.

**Conclusion**—Comprehensive genomic analysis suggests that thymic carcinomas are molecularly distinct from thymomas. These data have clinical, pathological, and therapeutic implications for the treatment of thymic malignancies.

### Keywords

Thymoma; Thymic Carcinoma; *EGFR*, *RAS* mutations; *KIT* mutations; mutational profiling; genomic analysis

#### Statement of translational relevance

Thymomas and thymic carcinomas are rare intrathoracic cancers that can be aggressive and refractory to conventional treatment. To identify potential targets for therapy, we performed a comprehensive molecular analysis of 45 thymic tumors. We found that molecular distinctions exist between different histologic types of thymic tumors. For example, compared to thymomas, thymic carcinomas display many more chromosomal gains and losses and exclusively harbor somatic mutations in the kinase encoded by *KIT*. Corresponding *KIT* mutants studied biochemically displayed sensitivity to the *KIT* inhibitors, imatinib and sunitinib. Some thymic malignancies harbor mutations in *RAS* genes, which have been previously associated with resistance to *EGFR*-directed therapies. These results have direct therapeutic implications for the treatment of thymic malignancies.

### Introduction

Thymomas and thymic carcinomas are malignant intrathoracic tumors which represent about 0.2% to 1.5% of all malignancies [1]. In general, thymomas are tumors with a tendency toward local recurrence rather than metastasis. Thus, most thymomas are treated surgically followed possibly by radiation [2]. By contrast, thymic carcinomas have a high risk of relapse and death despite surgery, chemotherapy, and radiation [3]. The optimal treatments for thymic tumors are not well-defined.

Because thymomas and thymic carcinomas are rare and both arise from thymic epithelium, they are often grouped together clinically. At the pathologic level, tumors of the thymus are classified according to criteria put forth by the World Health Organization (WHO) in 2004 [4]. In this schema, thymic epithelial malignancies are classified into thymomas (types A, AB, B1, B2, B3) and thymic carcinoma. These classes are based upon the morphology of epithelial cells (with an increasing degree of atypia from type A to thymic carcinoma), the relative proportion of the non-tumoral lymphocytic component (decreasing from types B1 to B3), and resemblance to normal thymic architecture [4]. Clinically, the degree of invasion or tumor stage is generally thought to be an important indicator of overall survival [5]. The best prognostic factor, however, is whether the tumor can be completely resected at the time of operation [6].

Compared to more common epithelial cancers, current knowledge about the biology of thymic tumors is limited. Research has been hampered by the rarity of the tumor and a lack of established cell lines and animal models. Recently, selected genes (*EGFR*, *KIT*, and *TP53*) have been analyzed in small cohorts of patients [7–12]. Notably, a number of cases have been reported of advanced thymic tumors responding to new targeted agents [13–17]. These cases suggest that thymic malignancies may be comprised of clinically relevant subsets that can be defined at the molecular level.

In order to gain insights into the biology of thymomas and thymic carcinomas, we performed a comprehensive genomic analysis of 45 resected thymic tumors. We used array comparative genomic hybridization, mutational profiling to assess the status of specific genes, mRNA expression profiling, and immunohistochemical analyses of proteins implicated in thymic tumor pathogenesis. We found differences between thymomas and thymic carcinomas that have important clinical and therapeutic implications.

## Materials and Methods

### Patient samples

Tumors specimens from patients with resected thymoma or thymic carcinoma who underwent surgical resection at Memorial Sloan-Kettering Cancer Center (MSKCC) from January 1997 to December 2007 were obtained with patients' consent under institutional tissue procurement protocols. All tumors were frozen either in the operative room or in the Pathology Department and stored at  $-80^{\circ}\text{C}$  in institutional tumor banks.

Clinical stage was determined according to the Masaoka system [5]. Frozen specimens, as well as all additional pathologic material available from corresponding tumors, were reviewed by a single reference pathologist (W.D.T.) for tumor classification according to the 2004 WHO criteria [4]. For this study, we selected thymic tumors which, on matching frozen specimens, contained at least 50% epithelial cells. This cutoff was chosen to eliminate the need for microdissection of epithelial cells within tumor samples of mixed epithelial and lymphocytic subtypes, as reported previously [18]. Only B2 thymomas that met these criteria were included. We excluded from the study thymoma types AB and B1, because of the problem of extensive lymphocytes in the stroma, which could confound genomic analyses.

### Mutational Profiling

DNA was extracted using standard methods. Mutational profiling was done using mass spectrometry-based genotyping (Sequenom, Sequenom, San Diego, CA), as previously described [19]. All samples were analyzed for a total of 74 Sequenom assays, designed to detect 101 known somatic mutations in genes of the EGFR signaling pathway: *EGFR*, *KRAS*, *HRAS*, *NRAS*, *BRAF*, *PIK3CA*, *AKT1*, *ERBB2*, and *MEK1* (Supplemental Table 1) [20].

In addition, we performed direct dideoxynucleotide-based sequencing of select exons from genes known to be commonly mutated and for which Sequenom assays were not available: *EGFR* exon 19, *KIT* exons 9, 10, 11, 13, 14, 17, and all coding exons of *TP53* and *PTEN* (See Supplemental Table 2 and Supplemental Methods).

### Genomic profiling

DNA was digested and labeled by random priming using Bioprime reagents (Invitrogen, Carlsbad, CA) and Cy3- or Cy5-dUTP. Labeled DNA was hybridized to Agilent 244K comparative genomic hybridization (CGH) arrays (Agilent Technologies, Santa Clara, CA). Normal genomic DNA (Roche, Basel, Switzerland) was used as a reference for all samples. After washing, hybridized slides were scanned and images quantified using Feature Extraction 8.5 (Agilent Technologies). Data were interpreted using standard methodology (See Supplemental Methods).

### Expression profiling

RNA was extracted using standard methods. RNA was converted to double-strand cDNA using T7-promoter-tagged oligo d(T) primers and reverse transcriptase. RNA targets were synthesized from cDNA by *in vitro* transcription and then labeled with biotinylated UTP and CTP. Biotinylated cDNA was fragmented and hybridized for 16 hours at  $45^{\circ}\text{C}$  to HG-U133A

2.0 Affymetrix oligonucleotide arrays. Data were analyzed using standard methods (See Supplemental Methods).

As expression profiles may considerably be altered by induction chemotherapy, especially with anthracyclines, and/or radiotherapy, as previously reported for other tumor types [21], pretreated tumors were not analyzed by global gene expression arrays.

### Immunohistochemistry

EGFR immunohistochemistry (IHC) was done using anti-EGFR mouse antibody clone 31G7 (1:1000) from Invitrogen (Camarillo, CA) and antigen retrieval by pepsin at 0.5%. KIT staining was performed using an anti-KIT rabbit polyclonal antibody from Dako (A4502; 1:2000, Carpinteria, CA) on a Ventana Symphony instrument (Ventana, Tucson, AZ). The staining intensity of the epithelial tumor cells was qualitatively evaluated from 0 (no staining) to 1+ (low staining), 2+ (moderate), and 3+ (elevated staining) by 2 investigators (N.G. and M.F.Z.), who were blinded to clinical and genomic data. For EGFR, only membrane staining was evaluated. For KIT, either cytoplasmic or membrane staining was considered positive. CD5 expression results in thymic carcinomas were retrieved from patients' pathology reports, as this staining was done part of the routine pathology diagnosis using an anti-CD5 rabbit monoclonal antibody (Clone SP19, Ventana). Negative and positive controls were included in all studies.

### Ba/F3-KIT mutant transformant assays

Ba/F3 cells harboring the *KIT*<sup>V560del</sup> and *KIT*<sup>H697Y</sup> mutations were generated using the QuikChange II XL site-directed Mutagenesis Kit (Stratagene Agilent Technologies), as previously described (See Supplemental Methods) [22]. For drug sensitivity assays, *KIT*<sup>V560del</sup> and *KIT*<sup>H697Y</sup> cells were incubated with 10 to 10,000 nM imatinib mesylate (provided by Novartis, Basel, Switzerland) or sunitinib malate (provided by Pfizer, New York, NY) in a 96-well plate in triplicate at the density of  $1 \times 10^5$  cells/well in 100  $\mu$ l of media for 48 hours (37°C, 5% CO<sub>2</sub>); 20  $\mu$ l of the tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] or Cell Titer Blue Reagent (Promega, Madison, WI) was then added and incubated with cells for 2 hours. For MTS assays, the absorbance at 490nm was recorded with a DTX880 multimode detector (Beckman Coulter, Fullerton, CA). For Cell Titer Blue, the fluorescence was read with a Spectra Max M5 detector (Molecular Devices, MDS, Toronto, Canada). Cell growth-inhibition was plotted as the ratio of the average quantity of formazan product (MTS) or resazurin reduction (Cell Titer Blue Reagent) in treated wells relative to non-treated controls. Assays were repeated at least 3 times in triplicate. The GI<sub>50</sub> values for imatinib and sunitinib were calculated using a standard non-linear regression algorithm available in the GraphPad Prism software (GraphPad, La Jolla, CA), version 4.03.

### Statistical methods

All patients were included in the statistical calculations. Follow-up was obtained in all cases, and was censored on June 30<sup>st</sup>, 2009. Categorical variables were compared using the Chi-square test. Survival was assessed using the Kaplan-Meier method. Results were considered significant at the 0.05 level. Statistical analyses were performed using the SPSS software program (Chicago, IL), version 17.0.

## Results

### Patient characteristics

A total of 98 thymic tumor specimens were stored in MSKCC tumor banks during the study period (Supplemental Figure 1). 64 cases corresponded to previously untreated tumors, which were resected upfront after diagnosis. 34 tumors were resected after induction treatment. For this study, we identified 45 eligible thymic tumor tissue specimens, 27 from previously untreated tumors and 18 from pre-treated tumors (Supplemental Figure 1). The clinical characteristics of patients with examined tumors are listed in Supplemental Table 3. 23 thymic tumors occurred in men, and 22 in women. The median age at diagnosis was 62 years. Tumor type was thymoma (types A, B2, B3) in 38 cases, and thymic carcinoma in 7 cases. At the time of analysis, all but 3 patients were alive. Median follow-up was 31.4 months (range 1.4–160.0 months). Five-year survival was 92%, and median survival was not reached.

### Exploration of the EGFR signaling pathway

At least one patient with thymoma has been reported in the literature to have had a documented radiographic response to the EGFR tyrosine kinase inhibitor (TKI) erlotinib [16]. Because mechanisms of sensitivity and resistance to EGFR inhibitors have been well-defined, we first investigated the status of various EGFR pathway biomarkers.

Using IHC with an anti-EGFR antibody, we found low intensity staining in 6 (15%) cases, moderate staining in 10 (26%) cases, and high staining in 23 (51%) cases. 6 cases were not interpretable (Table 1). There was no strong correlation between EGFR staining and thymoma histological type. High EGFR staining was significantly associated with stage III-IV tumors ( $p=0.023$ , Chi-square test).

We then profiled tumors for the presence of mutations in genes encoding components of the EGFR signaling pathway that are known to be mutated at specific recurrent nucleotide positions in human cancers [20]. Somatic *RAS* mutations were found in 3 (7%) of 45 tumors (Table 2): a heterozygous G to C substitution at nucleotide position 35 in exon 1 of *KRAS*, resulting in an alanine for glycine amino acid substitution at position 12 (G12A) in a type B2 thymoma (case 28); a heterozygous G to T substitution at nucleotide position 35 in exon 1 of *KRAS*, resulting in a valine for glycine amino acid substitution at position 12 (G12V) in a thymic carcinoma (case 41); and a heterozygous G to T mutation at position 38 in exon 1 of *HRAS*, resulting in a valine for glycine amino acid substitution at position 13 (G13V), in a type A thymoma (case 3) (Figure 1). EGFR staining was high in the *KRAS*-mutant tumors and moderate in the *HRAS*-mutant tumor (Figure 1). We did not detect any mutations in the *EGFR* kinase domain or in any other tested EGFR signaling pathway genes.

### Exploration of KIT signaling pathway

Patients with thymic tumors have exhibited radiographic responses to KIT inhibitors like imatinib [13–15]. Moreover, KIT staining by IHC has been reported to be frequent in thymic carcinoma [7,8,23,24]. Thus, we next investigated the status of KIT.

Three (7%) of 45 tumors displayed staining with an anti-KIT antibody (the same antibody used to assess KIT expression in GastroIntestinal Stromal Tumors (GISTs)), all of which were thymic carcinomas (Table 1). Direct sequencing of exons that encode the *KIT* kinase and transmembrane domains revealed that 2 (4%) of the 45 tumors, both thymic carcinomas, harbored *KIT* mutations (Table 2). One tumor, CD5-positive, contained a heterozygous deletion of nucleotides 1678 to 1680 in exon 11 of *KIT*, resulting in a deletion of valine at position 560 (V560del) (Figure 1). The other mutation, found in a CD5-negative tumor, was a heterozygous C to T mutation at position 2089 in exon 14 of *KIT*, resulting in a tyrosine for

histidine amino acid substitution at position 697 (H697Y) (Figure 1). Both *KIT* mutations were somatic. *KIT* mutations were mutually exclusive with *RAS* mutations.

The *KIT* V560 deletion occurs in the juxta-membrane domain of the *KIT* protein. Similar types of mutations (i.e. *KIT* V560G, *KIT* del557-558) have been found in GISTs sensitive to imatinib [25], and this exact mutation was previously observed in a case of thymic carcinoma responding to imatinib [13]. The *KIT* H697Y has not been previously reported in any cancer (COSMIC). The H697 residue is part of the *KIT* “kinase insertion domain” [26], a region that does not show amino-acid sequence homology with other kinases (Figure 2E). The region was not visualized in the published crystal structure of *KIT* [26]. However, this residue is conserved through *KIT* orthologs (Supplemental Figure 2), suggesting that it is a critical residue for *KIT* protein function.

To confirm that the V560del mutation is associated with imatinib sensitivity, and to study the H697Y mutation, we generated stable Ba/F3 transfectants expressing the mutant alleles. Ba/F3 cells are a murine pro-B cell line that are dependent on IL-3 for growth and which can be rendered IL-3-independent after the introduction of transforming kinase oncogenes [27]. Ba/F3-*KIT*<sup>V560del</sup> and Ba/F3-*KIT*<sup>H697Y</sup> cells grew in the absence of IL-3, demonstrating that these mutations confer gain-of-function (data not shown). The growth of Ba/F3-*KIT*<sup>V560del</sup> cells was potently inhibited by imatinib and sunitinib with GI<sub>50</sub>s of 15.0nM and 13.6 nM, respectively (Figures 2A and 2B). The growth of Ba/F3-*KIT*<sup>H697Y</sup> cells was more potently inhibited by sunitinib (GI<sub>50</sub> of 13.2 nM) than by imatinib (GI<sub>50</sub> of 631.4 nM) (Figures 2C and 2D). Although the latter GI<sub>50</sub> was still below 1000nM for imatinib, a cut-off reported for resistance to this drug in GISTs [22,28], these data suggest that sunitinib may be a more effective *KIT* inhibitor than imatinib in *KIT*-mutant thymic carcinomas.

### Gene expression profiling

We next performed mRNA expression profiling of 23 out of the 27 previously untreated thymic tumors (those with adequate high quality RNA for hybridization to Affymetrix U133A oligonucleotide arrays). Normal human thymus tissue was unavailable for analysis. Unsupervised hierarchical cluster analysis of gene expression data revealed 2 major groups (Figure 3): cluster 1 (n=8) and cluster 2 (n=15). Cluster 1 was associated with thymoma type B2 histology, while cluster 2 was associated with thymoma type A and thymic carcinoma ( $p=0.023$ , Chi-square test). Gene Ontology based pathway analysis revealed that most of the genes that were more highly expressed in cluster 1 vs. cluster 2 were related to the immune system (Supplemental Tables 4, 5 and 6). This result may be due to the lymphocytic infiltrates found in type B2 thymomas compared to the epithelial predominance of type A thymomas and thymic carcinomas, although clustering of the B2 thymomas was not correlated with differences in epithelial tumor cell content of the analyzed specimens. Cluster 2 could be further separated into 2 groups, clusters 2A (n=6) and 2B (n=9), which were associated with thymic carcinoma and thymoma type A, respectively ( $p=0.036$ , Chi-square test). Overall, the hierarchical clustering analysis correlated well with the histologic WHO classification. There were no associations with clinical stage, survival, EGFR/*KIT* staining or mutation. We did not identify genes encoding kinases as being overexpressed in any type or cluster of tumors, including *RAS*- and *KIT*-mutant tumors.

### Genomic profiling

To identify potential recurrent copy number changes at the DNA level, we performed array-based comparative genomic hybridization using Agilent 244K arrays and DNA from the 45 thymic tumor specimens. A hierarchical clustering algorithm generated 2 distinct clusters (Figure 4A). Cluster 1 (n=19) was associated with thymic carcinoma and type B3 thymoma and characterized by multiple chromosomal aberrations, whereas cluster 2 (n=26) was

associated with type A and B2 thymomas ( $p < 0.001$ , Chi-square test) and showed infrequent copy number alterations. There was no association between clinical stage ( $p = 0.065$ , Chi-square test) or neoadjuvant treatment ( $p = 0.311$ , Chi-square test) and these genomic clusters. Cluster 1 further subdivided in 2 major clusters, cluster 1a ( $n = 9$ ) with only type B3 thymomas and thymic carcinomas, characterized by chromosome 1q gain, and cluster 1b ( $n = 7$ ), with thymomas and thymic carcinomas sharing chromosome 6 loss (Figure 4A). Cluster 1 was significantly associated with high EGFR expression at IHC, as compared with cluster 2 ( $p = 0.001$ , Chi-square test).

We separately analyzed thymoma types A, B, and thymic carcinoma, to identify recurrent gene copy number alterations (CNAs) (Figure 4B and Supplemental Table 7). Only thymic carcinoma exhibited CNAs that were not reported as copy number variations. No recurrent CNAs occurred in *KRAS*, *HRAS*, or *KIT*, or in genes differentially expressed in the different tumor types.

### Comparison of carcinoma of the thymus vs. squamous cell carcinoma of the lung

Thymic carcinomas often display squamous cell histology and invade the lungs, pleura, and mediastinum [3]. As primary squamous cell carcinomas of the lung share many of these properties, mediastinal squamous cell carcinomas often present a diagnostic challenge. Thus, we asked whether molecular analyses could be used to discriminate these 2 entities. We directly compared the genomic profiles of the 7 thymic carcinoma tumors with those from 6 primary squamous cell carcinomas of the lung, that were matched for histological grade (Supplemental Table 8). CD5 was expressed in 4 (57%) of the 7 thymic carcinomas (Supplemental Table 8). All tumors, including CD5-negative cases, demonstrated classic diagnostic, clinical, pathological, and surgical features of the disease. Copy number data analysis revealed that compared to squamous lung cancers, chromosomal aberrations were more frequent and occurred in different and larger loci in thymic carcinomas (Supplemental Figure 3). Thymic carcinomas exhibited gains in chromosomes 1, 3q, 5, 8, 12, 15, 17q, 18, and 20, and losses in chromosomes 1p, 2p, 3p, 6, 7, 13q, 14, 16q, and 17p losses. Lung carcinomas displayed chromosome 2q, 3q, 5p, 7p, 8, 11q, 14p, 15p, 17p, and 18p gains, and chromosome 1q, 3p, 4p, 5q, 6q, 9p, 13q, 15p, 16q, and 17q losses (Supplemental Figure 3).

### Discussion

In order to gain further insight into the biology of thymic tumors, we performed a comprehensive molecular analysis of 45 resected thymic tumors. To our knowledge, this study represents the first and one of the largest of its kind. Previous studies have focused on only a small number of tumors [29] and employed only a limited number of analytical methods [29–31]. Our analysis has three main new findings.

First, mutational analysis of genes encoding components of the EGFR signaling pathway led to the identification of *RAS* mutations in 3 (7%) out of 45 thymic epithelial tumors. Previously, investigators have collectively assessed the status of only one *RAS* gene (i.e. *KRAS*) in 17 tumors [16,32,33], but did not identify any mutation. In our study, 2 *RAS* mutant tumors were thymomas (type A and B2) which are low-grade, and the other tumor was a thymic carcinoma, which is high-grade. *KRAS* mutations predict for primary resistance to anti-EGFR directed therapies (i.e. gefitinib/erlotinib in lung cancer [34] and cetuximab in colon cancer [35]). *HRAS* mutations occur much more rarely in epithelial cancers [36]. Cells expressing activated or mutant *HRAS* (T24 cell line [37]) have also been reported to be resistant to gefitinib [38, 39]. These findings could then have therapeutic implications for the treatment of thymic tumors. Although *RAS* mutations are rare in thymic tumors, further assessment of *RAS* is needed and should probably be included in any therapeutic trial considering anti-EGFR therapy for thymic malignancies.

None of the 45 tumors harbored *EGFR* kinase domain mutations, which are associated with sensitivity to gefitinib/erlotinib. Thus far, only 2 such mutations have been found in a total of 122 tumors collectively analyzed [7,9,10,16,33]. Coupled with the data on *RAS*, these findings could explain why responses to EGFR TKIs have been rare in unselected thymic tumors [33, 40]. As both *RAS* mutant tumors were strongly positive for EGFR staining, the data also suggest that IHC staining for EGFR is not useful as an eligibility marker for anti-EGFR therapies.

The second finding is the presence of *KIT* mutations in 2 of 45 thymic tumors. One was a V560 deletion in exon 11, and the other was a novel H697Y mutation in exon 14. In contrast to *RAS* mutations, *KIT* mutations were found exclusively in thymic carcinomas. Previously, out of 106 thymic tumors collectively tested, only 3 cases of *KIT* mutation have been reported in the literature [7,8,11,13,15,41,42]; all these mutations also occurred in thymic carcinomas. Interestingly, one was the same *KIT* V560 deletion found here. This mutation is associated with sensitivity to KIT inhibitors, based upon multiple lines of evidence: 1) we showed *in vitro* that the growth of mutant-bearing Ba/F3 cells is readily inhibited by treatment with imatinib and sunitinib; 2) the patient whose tumor harbored this mutation responded to treatment with imatinib [13], and 3) this mutation has also been found in an imatinib-sensitive GIST [43]. Another *KIT*-mutant case of thymic carcinoma reported in the literature harbored an L576P mutation in exon 11 [7]. This mutation has also previously been described in GIST and melanoma and has been biologically characterized as being sensitive to imatinib [44]. The third mutation reported is a D820E mutation in exon 17, exhibited by a thymic carcinoma responding to sorafenib [42]. The D820Y *KIT* mutation is known to be associated with imatinib resistance and sorafenib sensitivity in GIST [22]. Finally, we show here that the *KIT* H697Y mutation, identified in exon 14 (which was not sequenced in previous studies) is associated *in vitro* with greater sensitivity to sunitinib vs. imatinib.

In our series, using the same antibody for KIT IHC as used by others, 3 of 7 thymic carcinomas were positive, but only 2 harbored *KIT* mutations. The rarity of *KIT* mutations in unselected thymic tumors and the greater sensitivity of the H697Y mutant to sunitinib could explain the absence of responses observed in 2 recent phase II trials with imatinib, where patients were selected either by histologic type (B3 thymomas and thymic carcinomas) [41], or using KIT staining by IHC [45]. Overall, including our cases, 7% (5 out of 70 collectively genotyped) of thymic carcinomas exhibited *KIT* mutations. Similar to melanomas where *KIT* mutations are found in only 10% of tumors [46], we would recommend for future trials in thymic carcinomas with KIT inhibitors enrolling only those patients with *KIT* mutant tumors. We would also favor the use of sunitinib over imatinib.

Our genomic profiling data confirm previously reported analyses using lower resolution techniques of CGH [18,30,31]. Losses of chromosomes 1q, 3p, 17p and gains of chromosome 1q are found both in type B2-B3 thymomas and thymic carcinomas. Chromosome 6q, 13q, 16q losses and chromosome 4p, 17q and 18 gains are common in type B3 thymomas and thymic carcinomas [18,30]. As shown in Figure 4, the frequency, extent, and number of genomic aberrations increase from type A thymoma to thymic carcinoma. However, the clustering of a previous cohort of 65 tumors analyzed by CGH led to the identification of 2 groups, one sharing chromosome 1q gain and one with no recurrent pattern of chromosome imbalances [30]. The first group could be further subdivided into 2 clusters, one with chromosome 6q and 16q losses, and one with different genomic alterations [30]. In our cohort, cluster 1 subdivided in cluster 1a with chromosome 1q gain and cluster 1b with chromosome 6 and, to a lesser extent, chromosome 16q losses (Figure 4). The significance of these genomic alterations is unknown.

By using high resolution techniques of molecular profiling, our objective was also to identify genes or chromosomal regions that would have been altered both at the genomic and expression levels, i.e. overexpressed and amplified or underexpressed and deleted. However, we did not



identify such regions, especially involving genes encoding proteins with therapeutic significance, such as tyrosine and serine-threonine kinases. The lack of findings could be due to the low number of samples analyzed. We also were not able to compare expression data from tumors with that from matched normal thymus samples, which was unavailable, as in other studies [29,47]. Finally, especially for type B2 thymomas, results may have been confounded by the presence of lymphocytic infiltrates, although we did not identify any differences in the profiles depending on the epithelial tumor cell content.

One study previously reported results from gene expression profiling in thymomas [29,47]. Only 4 tumors were actually analyzed: 2 non invasive tumors (one stage I type A thymoma, and one stage II type B3 thymoma), and 2 invasive tumors (2 stage IVa type B3 thymomas). Among several genes, the authors identified *C-JUN*, *KIAA0022*, and *GPI-80* as being significantly correlated with tumor stage. We compared each tumor type one to another for differentially expressed genes (data not shown), but we did not identify these genes in our analyses.

Collectively, our expression and genomic clustering results indicate that tumors of the thymus defined histopathologically according to the current 2004 WHO classification do have different molecular features. Expression profiling separates type B2 thymomas, containing biologically active lymphocytes, from other thymic tumors, which are mostly epithelial. Furthermore, genomic profiling clearly discriminates thymic carcinomas and type B3 thymomas from type A and type B2 thymomas. From a classification perspective, our analysis then supports the distinctions of type A, type B2, type B3, and thymic carcinoma. Consistent with the notion of subtypes, only the latter tumor type harbors *KIT* mutations. Collectively, these results imply that the molecular basis for thymomas and thymic carcinomas is unique.

Finally, our data provides the first direct molecular comparison of thymic carcinoma with lung squamous cell carcinoma further demonstrating that these tumors are molecularly distinct from squamous cell carcinomas of the lung. Genomic profiling data are consistent with previous indirect comparisons between the 2 entities [4], with 1q, 17q, and 18 gains and 3p, 6, 16q, and 17p losses in thymic carcinomas, and 3q, 11q, and 8q gains, and 3p, 5q, 9p, 13q losses in lung carcinomas. We were also able to identify additional differential alterations present in thymic tumors (chromosome 12 gains, and chromosome 2p, 6, 7, 14 losses). Consistent with these findings, we did not identify mutations in *TP53* in thymic carcinomas, although such mutations are frequent (55%) in lung squamous cell carcinomas [48].

In the future, we plan to include cases of type AB and B1 thymomas, together with more tumors of all subtypes, to validate our current findings and to determine if proposals to simplify the current WHO thymoma classification also have biological relevance [49,50]. We also plan to perform more comprehensive mutational profiling of thymic malignancies. In the meantime, we recommend that despite occurring infrequently, thymic carcinomas should be treated separately from thymomas for clinical and therapeutic considerations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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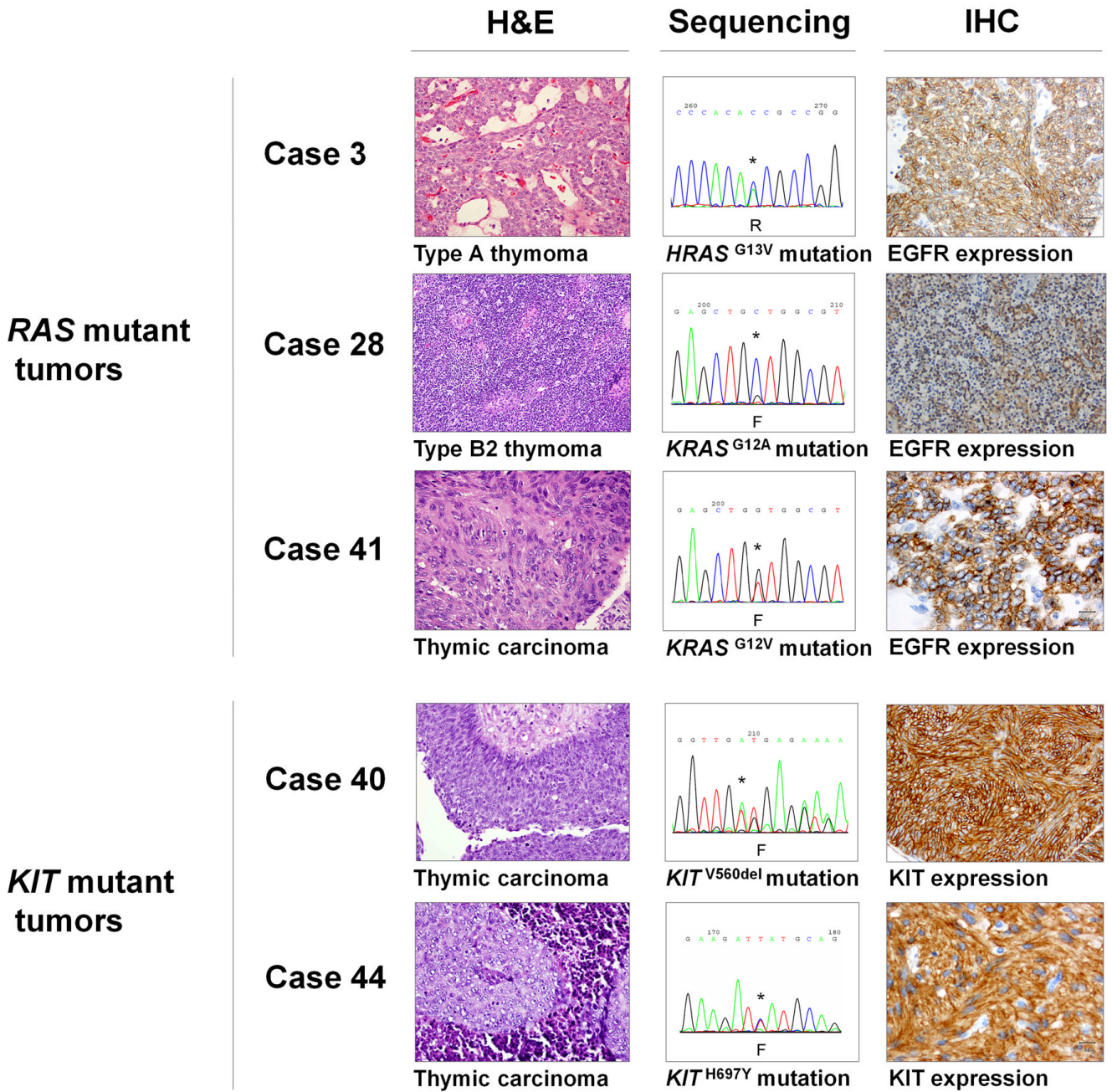
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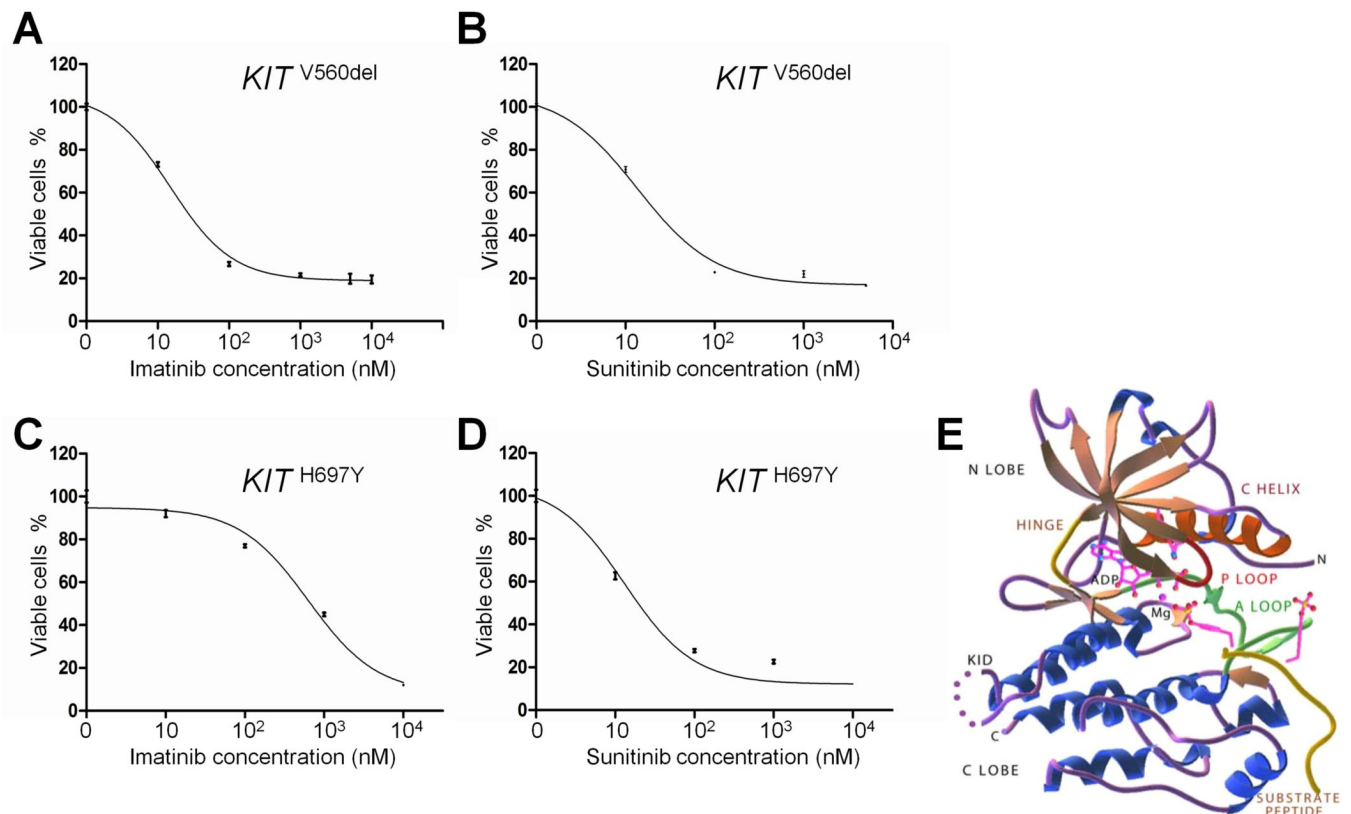
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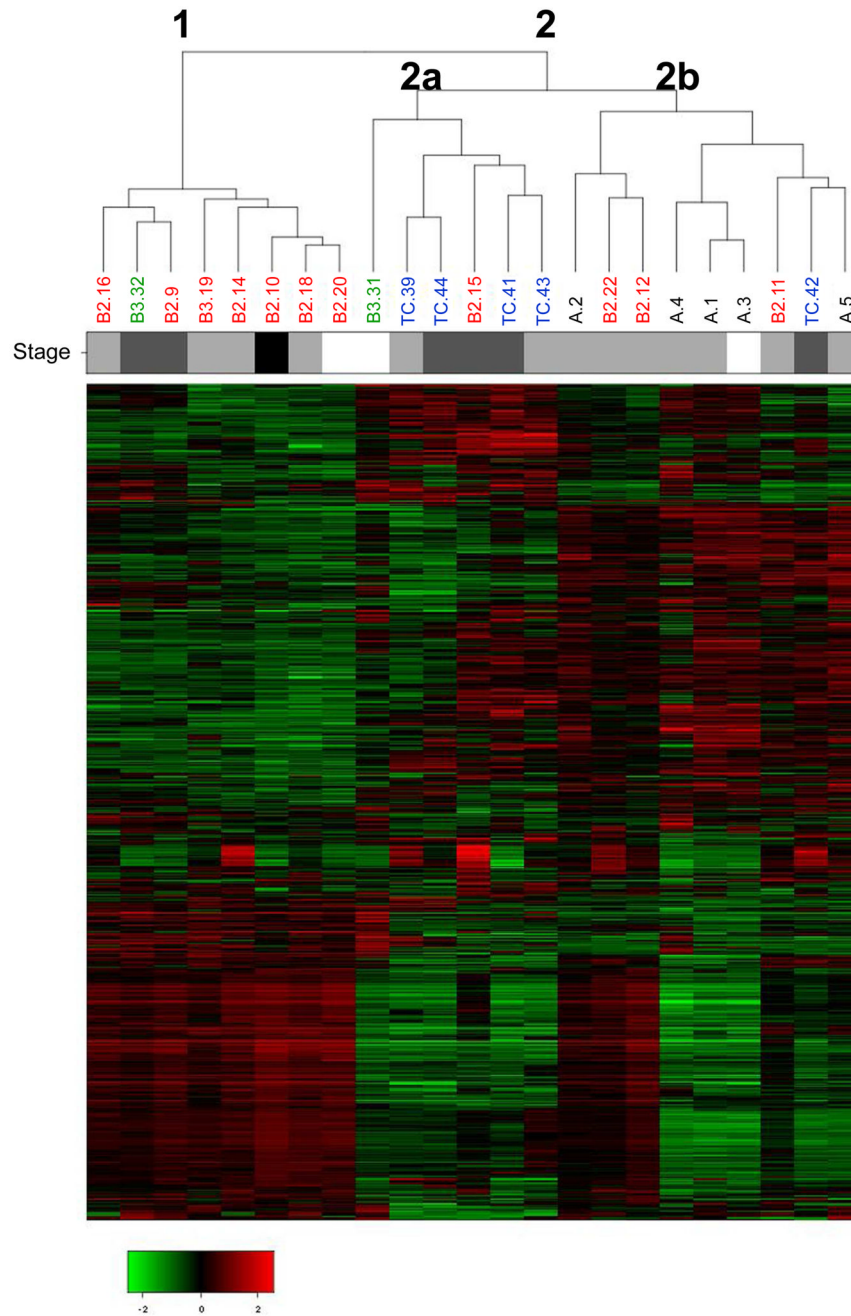
**Figure 1. RAS and *KIT* mutant thymic tumors**

Left panels: Hematoxylin and Eosin (H&E) stainings (original magnification,  $\times 40$ ). Middle panels: sequencing chromatograms. Right panels: immunohistochemical studies (IHC) with anti-EGFR or anti-*KIT* antibodies. The asterisks indicate the mutations.



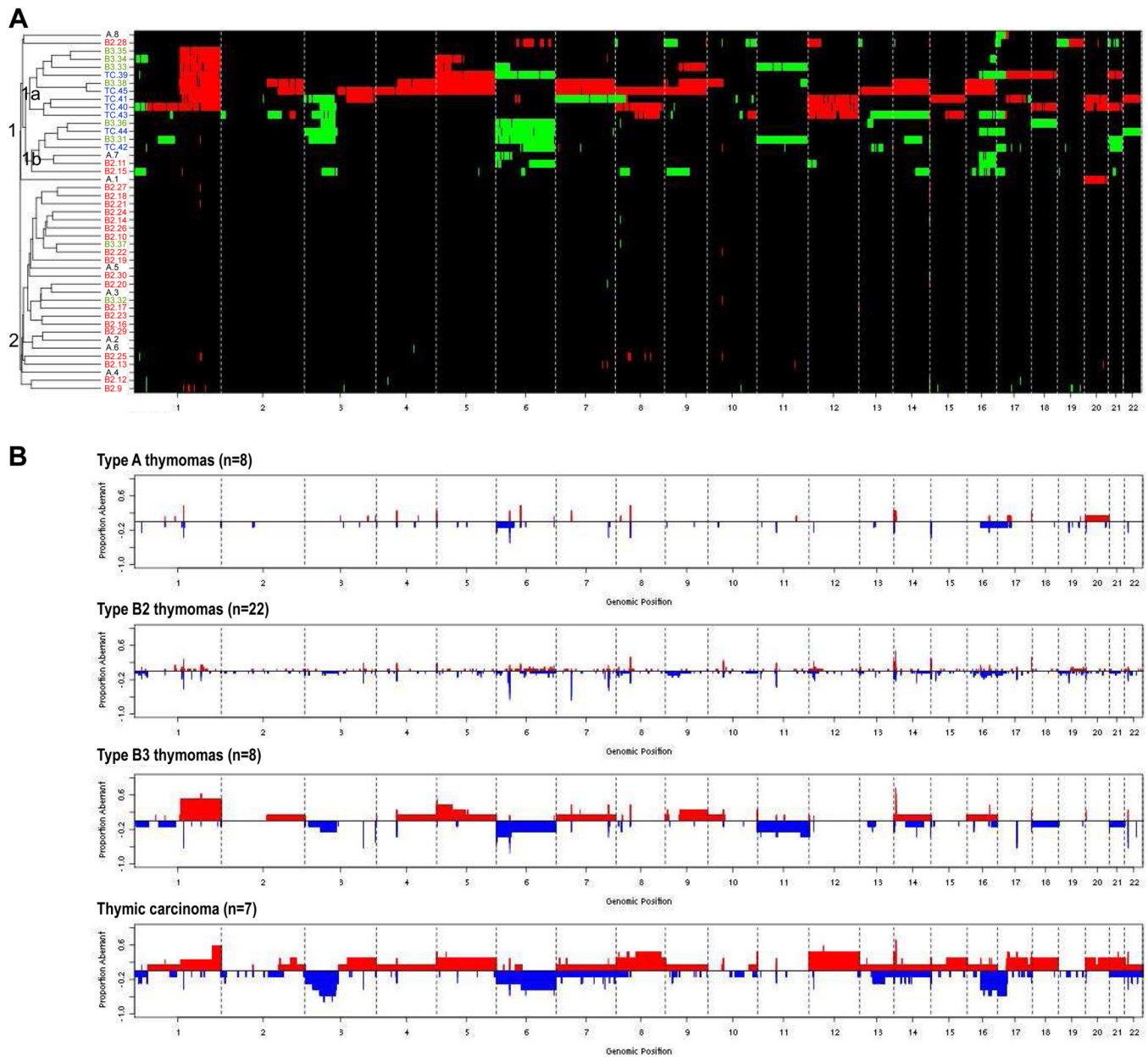
**Figure 2. Characterization of KIT mutants**

(A, B) The growth of Ba/F3-*KIT*<sup>V560del</sup> cells was potently inhibited by imatinib and sunitinib with GI<sub>50</sub>s of 15.0nM, and 13.6 nM, respectively. (C, D) On the contrary, Ba/F3-*KIT*<sup>H697Y</sup> cells were more sensitive to sunitinib (GI<sub>50</sub> of 13.2 nM), than to imatinib (GI<sub>50</sub> of 631.4 nM). (E) Crystal structure of KIT (from [26]). The *KIT* V560 deletion occurs in the juxta-membrane domain of the KIT protein. The H697 residue is located in the KIT “kinase insertion domain” (KID).



**Figure 3. Gene expression analysis of 23 thymic tumors**

Expression levels are colored red for values above the median, and green for values below the median. Type is indicated for each sample (TC: thymic carcinoma). For stage, white corresponds to stage 1, light gray to stage 2, dark gray to stage 3, and dark to stage 4.



**Figure 4. Genomic profiles of 45 thymic tumors**  
 (A) Unsupervised clustering analysis. Gains are indicated in red, and losses in green, by genomic position along the 22 chromosomes. (B) Genomic profiles and recurrent copy number alterations in type A and B thymomas, and in thymic carcinomas. Gains are indicated in red and losses in blue.



Table 1

Results of immunohistochemical stainings for EGFR and KIT. The intensity of the epithelial tumor cell staining was qualitatively evaluated from 0 (no staining) to 1 (low staining), 2 (moderate), and 3 (elevated staining).

Tumor	Pre-treated	Histology	Pathology Type	Stage	EGFR	Immunohistochemistry	KIT
1	no	Thymoma	A	2	NE		NE
2	no	Thymoma	A	2	NE		NE
3	no	Thymoma	A	1	2		0
4	no	Thymoma	A	2	1		0
5	no	Thymoma	A	2	3		0
6	yes	Thymoma	A	3	3		0
7	yes	Thymoma	A	3	NE		NE
8	yes	Thymoma	A	3	3		0
9	no	Thymoma	B2	3	2		0
10	no	Thymoma	B2	3	1		0
11	no	Thymoma	B2	4	3		0
12	no	Thymoma	B2	2	3		0
13	no	Thymoma	B2	2	2		0
14	no	Thymoma	B2	1	1		0
15	no	Thymoma	B2	2	2		0
16	no	Thymoma	B2	3	3		0
17	no	Thymoma	B2	2	1		0
18	no	Thymoma	B2	2	3		0
19	no	Thymoma	B2	2	2		0
20	no	Thymoma	B2	1	1		0
21	no	Thymoma	B2	2	3		0
22	no	Thymoma	B2	2	3		0
23	yes	Thymoma	B2	2	2		0
24	yes	Thymoma	B2	3	3		0
25	yes	Thymoma	B2	3	2		0
26	yes	Thymoma	B2	3	3		0
27	yes	Thymoma	B2	3	2		0
28	yes	Thymoma	B2	4	3		0
29	yes	Thymoma	B2	3	2		0
30	yes	Thymoma	B2	4	NE		NE
31	no	Thymoma	B3	1	3		0
32	no	Thymoma	B3	3	3		0
33	yes	Thymoma	B3	4	3		0
34	yes	Thymoma	B3	4	3		0
35	yes	Thymoma	B3	4	3		0
36	yes	Thymoma	B3	4	NE		NE
37	yes	Thymoma	B3	4	3		0
38	yes	Thymoma	B3	3	3		0
39	no	Thymic carcinoma	SCC	2	NE		NE
40	no	Thymic carcinoma	SCC	4	3		3
41	no	Thymic carcinoma	SCC	3	3		0
42	no	Thymic carcinoma	SCC	3	3		0
43	no	Thymic carcinoma	SCC	2	3		0
44	no	Thymic carcinoma	SCC	3	3		3
45	yes	Thymic carcinoma	SCC	3	2		3

Legend: SCC: Squamous Cell Carcinoma; NE: not evaluable.

Table 2

Results of mutational analyses.

Tumor	Pre-treated	Histology	Pathology Type	Stage	KRAS	Genes HRAS	KIT
1	no	Thymoma	A	2	wt	wt	wt
2	no	Thymoma	A	2	wt	wt	wt
3	no	Thymoma	A	1	wt	G13V	wt
4	no	Thymoma	A	2	wt	wt	wt
5	no	Thymoma	A	2	wt	wt	wt
6	yes	Thymoma	A	3	wt	wt	wt
7	yes	Thymoma	A	3	wt	wt	wt
8	yes	Thymoma	A	3	wt	wt	wt
9	no	Thymoma	B2	3	wt	wt	wt
10	no	Thymoma	B2	4	wt	wt	wt
11	no	Thymoma	B2	2	wt	wt	wt
12	no	Thymoma	B2	2	wt	wt	wt
13	no	Thymoma	B2	1	wt	wt	wt
14	no	Thymoma	B2	2	wt	wt	wt
15	no	Thymoma	B2	3	wt	wt	wt
16	no	Thymoma	B2	2	wt	wt	wt
17	no	Thymoma	B2	1	wt	wt	wt
18	no	Thymoma	B2	2	wt	wt	wt
19	no	Thymoma	B2	2	wt	wt	wt
20	no	Thymoma	B2	1	wt	wt	wt
21	no	Thymoma	B2	2	wt	wt	wt
22	no	Thymoma	B2	2	wt	wt	wt
23	yes	Thymoma	B2	3	wt	wt	wt
24	yes	Thymoma	B2	3	wt	wt	wt
25	yes	Thymoma	B2	3	wt	wt	wt
26	yes	Thymoma	B2	3	wt	wt	wt
27	yes	Thymoma	B2	3	wt	wt	wt
28	yes	Thymoma	B2	4	G12A	wt	wt
29	yes	Thymoma	B2	3	wt	wt	wt
30	yes	Thymoma	B2	4	wt	wt	wt
31	no	Thymoma	B3	1	wt	wt	wt
32	no	Thymoma	B3	3	wt	wt	wt
33	yes	Thymoma	B3	4	wt	wt	wt
34	yes	Thymoma	B3	4	wt	wt	wt
35	yes	Thymoma	B3	4	wt	wt	wt
36	yes	Thymoma	B3	4	wt	wt	wt
37	yes	Thymoma	B3	3	wt	wt	wt
38	yes	Thymoma	B3	3	wt	wt	wt
39	no	Thymic carcinoma	SCC	2	wt	wt	wt
40	no	Thymic carcinoma	SCC	4	wt	wt	V560del
41	no	Thymic carcinoma	SCC	3	G12V	wt	wt
42	no	Thymic carcinoma	SCC	3	wt	wt	wt
43	no	Thymic carcinoma	SCC	2	wt	wt	wt
44	no	Thymic carcinoma	SCC	3	wt	wt	H697Y
45	yes	Thymic carcinoma	SCC	3	wt	wt	wt

EGFR, AKT1, BRAF, ERBB2, MEK1, NRAS, PIK3CA, PTEN, and TP53 were wild-type (wt) in all tumors.

Legend: SCC: Squamous Cell Carcinoma