Amplification and Overexpression of the Dual-Specificity Tyrosine-(Y)-Phosphorylation Regulated Kinase 2 (DYRK2) Gene in Esophageal and Lung Adenocarcinomas

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ABSTRACT

Genomic amplification can lead to the activation of cellular proto-oncogenes during tumorigenesis, and is observed in most, if not all, human malignancies, including adenocarcinomas of lung and esophagus. Using a two-dimensional restriction landmark genome scanning technique, we identified five NotI/HinAI fragments with increased genomic dosage in an adenocarcinoma of the gastroesophageal junction. Four of these amplified fragments were matched within three contigs of chromosome 12 using the bioinformatics tool, Virtual Genome Scan. All three of the contigs map to the 12q13-q14 region, and the regional amplification in the tumor was verified using comparative genomic hybridization analysis. The 12q14 amplicon was characterized using sequence tagged site-amplification mapping with DNA from paired normal-tumor tissues of 75 gastroesophageal and 37 lung adenocarcinomas. The amplicon spans a region of >12 Mb between genes DGKA and BLOVI. The core-amplified domain was determined to be <0.5 Mb between marker WI-12457 and gene IFNG. However, MDM2, a well-documented oncogene of the region, is outside the core-domain. Eleven genes and expressed sequence tags within the amplicon were selected for quantitative reverse transcription-PCR, and DYRK2, a member of the dual-specificity kinase family, was overexpressed in all of the tumors showing gene amplification. Among the sequence tagged site/expressed sequence tag/gene markers tested, DYRK2 demonstrated the highest DNA copy number and the highest level of mRNA overexpression in the tumors. Moreover, DYRK2 mRNA overexpression (2.5-fold of normal mean) was found in 18.6% of additional 86 lung adenocarcinomas in an assay using oligonucleotide microarrays. DYRK2 mRNA overexpression occurs more frequently than gene amplification in both esophageal and lung adenocarcinomas. This is the first report of amplification and overexpression of DYRK2 in any tumor type.

INTRODUCTION

The incidence of esophageal adenocarcinoma has rapidly increased in many western countries including the United States, the United Kingdom, the Netherlands, Denmark, and Australia over the past 3 decades (1, 2). A poor overall 5-year survival rate of <10% portends the future clinical importance of this disease (3). Invasive adenocarcinoma is accompanied frequently with Barrett’s metaplasia, an intestinalized columnar epithelium that replaces the normal squamous epithelium at the distal esophagus as a result of chronic gastroesophageal reflux (4, 5). Smoking and obesity have been implicated as additional risk factors for the disease (6, 7).

Amplification of dominant-acting oncogenes plays a significant role in the development and/or progression of many types of human cancer, including esophageal and lung adenocarcinomas. We identified previously a novel amplicon at 8p22–23 in gastroesophageal adenocarcinomas (8). Using the STS-amplification mapping approach, the core-amplified domain was narrowed to include the cytoband p22 region, the transcription factor GATA-4 (9, 10). Similarly, we identified and characterized an amplicon at 19q12 and revealed cyclin E as the best candidate gene selected by the 19q12 amplification in gastroesophageal adenocarcinomas (10). In addition to oncogene activation via gene amplification, inactivation of the tumor suppressor gene p53 occurs frequently in esophageal adenocarcinoma (11, 12). Amplification and overexpression of the MDM2 oncogene were reported infrequently (4%) in another study in this type of tumor (16). Whereas involvement of p53 was observed frequently in esophageal adenocarcinomas, amplification of the MDM2 gene was not detected in one study (15) and was reported infrequently (4%) in another study in this type of tumor (16). Using CGH, Moskaluk et al. (17) and van Dekken et al. (18) reported increased 12q DNA copy number in 27% and 39% of esophageal adenocarcinomas. However, these regions of gain were mapped to 12q12-q24, suggesting an additional 12q amplon because MDM2 is mapped to the 12q13–15 region (13, 14). Tumors with amplified genes closely linked to MDM2 but without MDM2 amplification or with discontinuous amplons including MDM2 were also reported (20–22). To clarify the amplification status within the 12q region, the current investigation was undertaken using RLGS (23, 24) combined with recently developed bioinformatics tools, VGS (25). Use of these tools allows individual genomic fragments amplified in a tumor genome to be identified. DYRK2, a dual-specificity tyrosine-(Y)-phosphorylation regulated kinase gene, was identified as the most frequently amplified and overexpressed gene among the STS/EST/gene markers examined, and also demonstrated the highest mRNA overexpression level among the genes tested in this series of gastroesophageal and lung adenocarcinomas. Both lung and esophageal adenocarcinomas were examined in this study to determine whether the amplicon is tumor type-specific or whether it occurs in multiple tumor types. The present study represents the first complete characterization of the 12q14 amplification in both lung and esophageal adenocarcinomas, and is the first to report amplification and overexpression of DYRK2 in any human cancer.

MATERIALS AND METHODS

Tissue Collection. After obtaining written consent, 75 esophageal and gastric cardia adenocarcinomas, the corresponding normal squamous esophageal or gastric mucosa, and 123 lung adenocarcinomas were obtained from patients undergoing esophagectomy or pulmonary resection at the University of Michigan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: STS, sequence tagged site; RLGS, restriction landmark genome scanning; VGS, Virtual Genome Scan; CGH, comparative genomic hybridization; QG-PCR, quantitative genomic-PCR; EST, expressed sequence tag; NCBI, National Center for Biotechnology Information; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
of Michigan Medical Center between 1992 and 2000. Patients in this study had no preoperative radiotherapy or chemotherapy. A small portion of each tissue was embedded in OCT compound (Miles Scientific, Naperville, IL) and stored at −70°C; the remainder was stored at −70°C until use.

**DNA Isolation and RLGS-Combined VGS Analyses.** High molecular weight DNA was extracted as described previously (26) from tumors with >70% tumor cellularity. Two-dimensional RLGS was performed as described previously (27). In brief, DNA from normal and tumor tissues was double digested using the restriction enzymes NotI and EcoRV (New England Biolabs Inc., Beverly, MA), and NotI ends were filled with [α-32P]dCTP and [α-32P]dGTP (NEB Life Science Products, Boston, MA). First-dimensional size-fractioning was performed in 0.9% disk-agarose gel. The resulting DNA fragments were then in-gel digested with HinfI and separated in the second dimension on a 5.25% polyacrylamide gel. Gels were then dried and autoradiographic images obtained (Molecular Dynamics, Sunnyvale, CA). Digital images were analyzed using ImageQuant v1.2 software (Molecular Dynamics). Amplified DNA fragments in tumors were quantified by densitometry using the two-dimensional images from the corresponding normal tissue DNA as standards. The VGS bioinformatics tools1 were used to match the positive fragments identified by actual two-dimensional RLGS analysis as described previously (25). The chromosome 12 VGS image was captured and used to create Fig. 1C.

**CGH.** The CGH procedure was based on the protocol described previously with modifications (28). Briefly, 200 ng of DNA samples isolated from tumor and the corresponding normal tissues was labeled by nick translation with fluorescein-isothiocyanate-dUTP (green) and Texas-Red-dUTP (red), respectively. The probes were mixed with human Cot-1 DNA (10 μg), denatured, reannealed, and hybridized to methotrexate-synchronized male metaphase chromosomes on glass slides, and incubated at 37°C for 3 days under humid conditions. The preparations were washed to remove nonspecific bound DNA and counterstained with 4',6-diamidino-2-phenylindol for chromosome identification. Images were captured using a Zeiss Axiophot fluorescent microscope equipped with filters for CGH and analyzed using QUIPS software (Vysis, Downers Grove, IL). Data from 20 metaphase spreads were used to generate a composite CGH profile. Threshold values were 0.8 for loss and 1.2 for gain.

**STS-Amplification Mapping Using QG-PCR.** STS markers located in the region identified as having high-level amplification by CGH were selected for quantitative genomic PCR as described previously (9). PCR primers were designed to assure that the melting temperature (Tm) of each primer set was compatible with that of the internal control, herein GAPDH, which resides on the same chromosome 12 so the ratio of tumorsample/GAPDH: normalsample/GAPDH will reflect the actual DNA copy numbers increased in tumor. The quantity of the normal and tumor genomic DNA was measured by fluorometry (TKO100; Hoefer Scientific Instruments, San Francisco, CA) to ensure equity of the starting DNA. Forward primers for GAPDH and each STS/gene marker were end-labeled with [γ-32P]ATP (NEN Life Science Products) using T4 polynucleotide kinase (New England Biolabs). PCR was performed using Taq polymerase (Promega, Madison, WI), and the PCR products were resolved in 8% denaturing polyacrylamide gels. PCR product signal ratios for both the tumor (tumor STS fragment/tumor GAPDH fragment) and normal DNA samples (normal STS fragment/nominal GAPDH fragment) were quantified using ImageQuant software (Molecular Dynamics). Values ≥2.0 were considered indicative of DNA amplification. All of the results were verified by repeating

Table 1. Primer sequences of gene/STS markers used for QG-PCR analysis of the 12q14 amplicon

<table>
<thead>
<tr>
<th>Gene/STS</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
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<td>ggtttcttcctctctctcttc</td>
<td>gccttccactctcgctccag</td>
</tr>
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<td>agtgtgcagacggtccgcaag</td>
<td>gcagagaagctcggagcaggg</td>
</tr>
<tr>
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2 These primers can only be utilized for genomic PCR amplification.
three times. The primers for the STS/EST/gene markers analyzed in the present study are listed in Table 1.

**RNA Isolation and RT-PCR.** Total RNA was isolated using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). Agarose gel electrophoresis and the A_{260}/A_{280} ratio were used to assess RNA quality. RNA samples were treated with DNase I (Promega) before reverse transcription. Two μg of total RNA were incubated with reverse transcriptase (Invitrogen Life Technologies, Inc.) and primed by both (dT)_{18} and random hexamers in a total of 25 μl of reaction volume. cDNA (1.0–1.5 μl) underwent RT-PCR with GAPDH coamplified as an internal control. PCR products were then resolved on 8% PAGE gels, and gel data analyses were performed using ImageQuant software as in QG-PCR.

**Immunohistochemical Analysis.** Cryostat sections (5 μm) were placed on 0.1% poly-L-lysine-coated slides and fixed with 100% acetone at −20°C for 10 min. Endogenous peroxidase activity was quenched with three changes of 0.3% hydrogen peroxide for 30 min each. Horse serum, at a dilution of 1:20 in PBS-1% BSA, was used to block nonspecific binding. The MDM2 protein was detected using an anti-MDM2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution in PBS-1% BSA. Negative controls were incubated without primary antibody. Immunoreactivity was detected using the Vectastain avidin/biotin complex kit (Vector Laboratories, Burlingame, CA) and slides counterstained with Harris-modified hematoxylin.

**DYRK2 Expression Analysis in Lung Adenocarcinomas Using Oligonucleotide (Affymetrix) Microarray Data.** Total cellular RNA was isolated from 10 normal and 86 lung adenocarcinoma samples using TRIzol reagent.
RESULTS

Two-Dimensional RLGS and VGS of Gastroesophageal Adenocarcinomas. High molecular weight DNA from 44 primary esophageal and gastric cardia adenocarcinomas was analyzed using the two-dimensional RLGS technique. Approximately 3000 individual NotI/HinfI fragments can be visualized in a two-dimensional gel allowing global comparison of two genomes from normal and tumor tissues (Fig. 1). Comparison of the DNA from a gastroesophageal adenocarcinoma (tumor E13, Fig. 1B) with the DNA from its corresponding normal tissue (Fig. 1A) revealed 5 DNA fragments with increased intensity in the tumor tissue (Fig. 1B). VGS 1.01, a bioinformatics tool for comparison of sample-derived RLGS patterns with patterns predicted from the human genome sequence, was used. The amplified NotI/HinfI fragment B1 that was ~1359 bp in the first dimensional gel and 739 bp in the second dimensional gel (1359/739), B2 (1239/530), B3 (896/178), and B4 (1786/312) were matched to their respective virtual fragments C1 through C4, all located in chromosome 12 (Figs. 1 and 4). The sequences of matched virtual NotI/HinfI fragments were aligned within three chromosome 12 contigs, NT_029419, NT_009509, and NT_009458 (Table 2). A virtual fragment within the core-amplified domain around the D12S80 locus (Fig. 4). The core-amplified domain and boundary of the 12q14 amplicon in one tumor (B81, Fig. 1B) with the peak at 12q14 based on a composite CGH profile of 20 metaphase spreads (Fig. 2B). A genomic gain was also observed at chromosome 12p and 18p in the composite profile of the tumor (Fig. 2B).

Characterization of the 12q14 Amplicon Using STS-Amplification Mapping. To determine the core-amplified domain and boundaries of the 12q14 amplicon, >19 STS/EST/gene markers, spanning from the DGKA gene to D12S80 and covering ~15 Mb in the region, were selected from NCBI databases for QG-PCR analysis. The DNA from paired tumor and corresponding normal tissues of 75 gastrointestinal and 37 lung adenocarcinomas were screened with each of the markers using the QG-PCR assay (Fig. 3). QG-PCR results were verified at least two times. The 12q14 amplicon is >12 Mb with the centromeric cutoff at the DGKA gene and the telomeric border around the D12S80 locus (Fig. 4). The core-amplified domain is confined to a region <0.5 Mb between WI-12457 and IFNG (Fig. 4). In tumor G12, QG-PCR analysis indicated a possible low gain at the MDM2 locus. However, much higher values of increased DNA dosage (>5-fold) were consistently observed at DYSR2 and WI-12457 in the same tumor G12 (Fig. 3; Fig. 4A). WI-12457 is an EST fragment with high homology to TATA box binding protein-interacting protein (TIP120A) and is closely linked to DYSR2 (~300 Kbp). The core-amplified domain of the 12q14 amplicon is a continuous and common region between both gastrointestinal and lung adenocarcinomas (Fig. 4A). A separate regional amplification was found in the 12q13 region at the CDK2 locus in one tumor (B81, Fig. 3; Fig. 4A), suggesting an additional amplicon in the 12q13 region. MDM2 was not amplified in tumor G12. The tumor showing the highest DNA copy number of DYSR2, DYRK2 Is a Potential Candidate Gene Selected by the 12q14 Regional Amplification. Eleven genes, including those located within the core region of the 12q14 amplicon, were selected to

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determine RNA expression levels in the amplified tumors using quantitative RT-PCR (Fig. 5). DYRK2 was overexpressed in all of the amplified tumors and also in tumor B81, which was not amplified at the DYRK2 locus (Figs. 3 and 5). mRNA overexpression of the remaining 10 genes/ESTs tested, including MDM2, was also detected but none of them were overexpressed in all of the amplified tumors (Fig. 5; Table 3). Moreover, in the tumors, the level of DYRK2 mRNA overexpression was much greater than all of the other genes/ESTs located within the 12q14 amplicon including MDM2 (Fig. 5). Gene amplification and overexpression seemed to be confined only to DYRK2 when the other DYRK family members, DYRK1A, DYRK1B, DYRK3, and DYRK4, were examined and amplification and overexpression were not detected in this series of tumors (data not shown).

Although MDM2 is not included in the core-amplified domain in our series of adenocarcinomas (Fig. 3; Fig. 4A), we used an immunohistochemical assay to determine MDM2 protein expression because of the well-documented involvement of this gene in the p53 pathway in tumors. Tumors E13, G12, H38, and S01, the four tumors containing the 12q14 amplicon, were examined (Fig. 6, A–D). The 0SAS sarcoma cell line, which overexpresses MDM2, was used as a positive control (Fig. 6A). All of the negative controls showed no staining (data not shown). Lung adenocarcinoma H38 shows intense nuclear staining of MDM2 in a majority of tumor cells (Fig. 6B) compared with no staining in H38 normal lung (Fig. 6C). Tumor G12, which demonstrated only low gain in MDM2 copy number (Fig. 3), shows barely detectable nuclear staining in <5% of tumor nuclei (Fig. 6D). MDM2 staining was light and detected in <10% of tumor cell nuclei of tumor E13, and no nuclear staining was observed in lung tumor S01 (data not shown). DYRK2-specific antibodies are currently unavailable.

**Frequent Overexpression of DYRK2 mRNA in Lung Adenocarcinomas.** DYRK2 gene expression was analyzed using our oligonucleotide microarray database of 86 lung adenocarcinomas and 10 normal lungs (30). The gene expression data demonstrated a significant increase of DYRK2 expression in stage I lung tumors (P = 0.00088) and stage III tumors (P = 0.00073) as compared with the expression level in normal tissues. The difference of DYRK2 expression between stage I and III lung tumors was not found (P = 0.20). Array analysis of the expression patterns of the genes

*Fig. 4. Diagrammatic map of the 12q13-14 amplicon in gastroesophageal and lung tumors determined using QG-PCR analysis, and a map of the matched virtual NotI/HinfI fragments aligned to the actual amplification map. A, ● indicate that genomic amplification was detected in the individual tumors at the markers tested. ○ indicate that DNA amplification was not observed at the loci tested. The map order is based on QG-PCR analysis of 75 gastroesophageal and 37 lung adenocarcinoma tumors, but closely follows the December 2002 NCBI databases from various sources. As shown, the core-amplified domain (excluding tumor B81 of which the amplicon is centered on CDK2) is between the marker WI-12457 and gene IFNG, leaving MDM2 outside of the core-amplified region. Overexpression status of genes and ESTs in this region was analyzed, and DYRK2 was found to be the only gene overexpressed in all tumors amplified. B, the physical map of the matched virtual NotI/HinfI fragments as related to the actual amplification map shown at left.*
Table 3 Candidate search results using quantitative RT-PCR for amplified genes/ESTs located within the 12q13-14 amplicon.*

<table>
<thead>
<tr>
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<th>E13</th>
<th>G12</th>
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<th>S01</th>
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<tbody>
<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>ERBB3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAN1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td>IRAK-M</td>
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*The ratio of tumor/sample/GAPDH: normal/sample/GAPDH ≥ 2.0 is defined as gene overexpression. “+”, overexpressed; “−”, not overexpressed; “nt”, not tested.

was observed to be frequently amplified at high-levels (>30-fold) in sarcomas (33.3%) and gliomas (8–10%; Refs. 14, 33), our findings are consistent with two previous publications in which amplification of MDM2 was observed in only 0–4% of esophageal adenocarcinomas (15, 16). Infrequent or absent amplification of MDM2 was also documented in many other human cancers, including esophageal squamous carcinomas (0 of 24 tumors; Ref. 34), primary Ewing tumors (0 of 37 tumors; Ref. 35), primary cervical carcinomas (0 of 35 tumors; Ref. 36), breast cancers (1 of 60 tumors; Ref. 37), and hepatoblastomas (0 of 19 tumors; Ref. 38). The present study is the first to delineate the genes selected by the 12q14 amplification with close proximity to MDM2 in esophageal and lung adenocarcinomas.

The DYRK family includes dual-specificity tyrosine-(Y)-phosphorylation regulated kinases, which are distinguished from other protein kinase families by their ability to phosphorylate both Ser/Thr and Tyr substrates (dual-specificity), and includes at least seven mammalian members, DYRK1A, DYRK1B, DYRK1C, DYRK2, DYRK3, DYRK4A, and DYRK4B (41). In addition, DYRK1A, DYRK2, DYRK3, MNB, and YakA can autophosphorylate their tyrosine residues in vitro (39, 41). YakA may be the homologue of DYRK2 or DYRK3, and all three are predominantly cytoplasmic, whereas DYRK1A shows nuclear localization (40, 41). DYRK1A is the homologue of Drosophila MNB, which has been mapped to human Down syndrome critical region 21q22.2 (42, 43). Presently, the cellular function of all of the DYRK family members remains unclear. However, DYRK1A has been implicated in normal embryo neurogenesis (44) and as a potential candidate gene responsible for the Down syndrome (45). The DYRK family may also be involved in early heart development (46) and might play a role in cell cycle regulation (47). DYRK1A has been reported to phosphorylate the transcription factor FKHR at Ser329 in vitro (48). Both DYRK1A and DYRK2 have been shown to phosphorylate STAT3 in vitro (49). DYRK1A might be directly involved in regulating the transcriptional activity of Gli1, a transcription factor that is a target of the Sonic hedgehog pathway involved in basal cell carcinoma development (50–52). Interestingly, mirk, a recently cloned minibrain related kinase of which the cellular localization is similar to DYRK2 and DYRK3, is overexpressed in primary colon....

DISCUSSION

In the present study, DYRK2 demonstrated the highest level and frequency of amplification (Fig. 4A) and RNA overexpression (Table 3) among 19 STS/EST/gene markers examined in the 12q13-14 region. MDM2, on the other hand, was not included in the core-amplified domain in this series of 75 gastroesophageal and 37 lung adenocarcinomas (Fig. 3). Although the well-documented oncogene located within the 12q14 amplicon indicated that overexpression of DYRK2 in tumors acts independently from the rest of the genes closely linked (Fig. 6E), suggesting that DYRK2 is less likely a passenger of a closely linked gene, which is the driving force of the 12q14 amplicon. The frequency of DYRK2 mRNA overexpression (>2.5-fold of the normal mean expression) in this series of 86 lung adenocarcinomas is 18.6% (16 of 86) and is higher than the gene amplification frequency (5.4%) in the series of the 37 lung adenocarcinomas. Amplification analysis for these 86 lung tumors was not assessed because of finite amounts of tissue material. DYRK2 mRNA overexpression was also found in one esophageal tumor, B81, which did not contain DYRK2 gene amplification (Figs. 3 and 5). These data indicate that additional mechanism(s) other than gene amplification can also elevate DYRK2 expression in esophageal and lung adenocarcinomas.

**Fig. 6.** Immunohistochemical analysis of MDM2 protein expression in esophageal and lung tumors (A–D), and gene expression of DYRK2 in lung tumors using Affymetrix microarray analysis (E). A, the sarcoma cell line OSAS overexpresses MDM2 and was used as a positive control for nuclear staining (arrows). B, tumor H38, which contains the 12q14 amplicon, shows strong nuclear staining of MDM2 in most tumor cells (arrows). C, normal lung section from patient H38 displays no MDM2 staining. D, esophageal tumor from patient G12, which contains highly increased DYRK2 copy number but no detectable MDM2 amplification, shows only slight nuclear staining of MDM2. E, gene expression patterns sorted by DYRK2 expression levels for 86 lung adenocarcinomas and 10 normal lung samples using our array database (30) demonstrate substantially elevated expression (red) of DYRK2 in at least 16 individual tumors. The expression patterns of the other genes located within the 12q14 amplicon are also included, and the comparison of the expression patterns of these genes with the patterns of DYRK2 indicates that overexpression of DYRK2 in tumors is independent from other closely linked genes within the 12q14 amplicon.
cancerous and can mediate the survival of colon cancer cell lines under stress induced conditions (53). A recent study has reported that Dyrk2 mRNA was down-regulated in neuroblastoma cells by treating the cells with an anticancer agent, 8-Cl-CAMP, a site-selective cyclic AMP analogue exhibiting growth inhibition in a broad spectrum of human cancer lines (54). These observations, together with our findings, indicate a potential role of Dyrk2 in lung and esophageal tumor development and/or progression.

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REFERENCES


DYRK2 GENE AMPLIFICATION IN ESOPHAGEAL ADENOCARCINOMAS

Amplification and Overexpression of the Dual-Specificity Tyrosine-(Y)-Phosphorylation Regulated Kinase 2 (DYRK2) Gene in Esophageal and Lung Adenocarcinomas


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