Integrative Genomics Identifies RAB23 as an Invasion Mediator Gene in Diffuse-Type Gastric Cancer

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Abstract

Recurrent genomic amplifications and deletions are frequently observed in primary gastric cancers (GC). However, identifying specific oncogenes and tumor suppressor genes within these regions can be challenging, as they often cover tens to hundreds of genes. Here, we combined high-resolution array-based comparative genomic hybridization (aCGH) with gene expression profiling to target genes within focal high-level amplifications in GC cell lines, and identified RAB23 as an amplified and overexpressed Chr 6p11p12 gene in Hs746T cells. High RAB23 protein expression was also observed in some lines lacking overexpressed Chr 6p11p12 gene in Hs746T cells. High RAB23 migration in Hs746T cells, whereas overexpression of RAB23 amplification, suggesting additional mechanisms for up-regulating RAB23 besides gene amplification. siRNA silencing of RAB23 significantly reduced cellular invasion and migration in Hs746T cells, whereas overexpression of RAB23 enhanced cellular invasion in AGS cells. RAB23 amplifications in primary gastric tumors were confirmed by both fluorescence in situ hybridization and genomic qPCR, and in two independent patient cohorts from Hong Kong and the United Kingdom RAB23 expression was significantly associated with diffuse-type GC compared with intestinal-type GC (iGC). These results provide further evidence that dGC and iGC likely represent two molecularly distinct tumor types, and show that investigating focal chromosomal amplifications by combining high-resolution aCGH with expression profiling is a powerful strategy for identifying novel cancer genes in regions of recurrent chromosomal aberration. [Cancer Res 2008;68(12): 4623–30]

Introduction

Chromosomal instability is frequently observed in gastric cancer (GC), with up to 90% of primary tumors exhibiting aneuploidy (1). Previous studies have revealed a complex portrait of recurring chromosomal amplifications and deletions in GC, including gains and losses at chromosomes 1q, 8q, 17q, and 20q (2–4). The recurrent nature of these aberrations has been attributed to the presence of genes important for gastric carcinogenesis, such as CD44 at 11p13, CCNE1 at 19q13, and BTAK at 20q13 (5–7). However, identifying specific oncogenes and tumor suppressor genes within such regions is often challenging by their large size covering tens to hundreds of genes. For example, chromosome 6p amplifications have been reported in GC at frequencies ranging from 2% to 4% by comparative genomic hybridization (CGH) to 85% by cDNA-based array-based comparative genomic hybridization (aCGH; refs. 2, 8, 9), but specific genes representing the target of 6p amplification are currently unknown.

The importance of chromosomal aberrations in GC is also highlighted by the underlying genetics of its histologic subtypes. GCs are broadly classified into intestinal (iGC) or diffuse (dGC) forms (10), which exhibit strikingly different morphologies. iGCs form cohesive gland–like tubular structures, whereas dGCs exhibit minimal cell cohesion with frequent invasion into surrounding tissues (11). Although amplifications at 17q12-21 and 20q and losses at 8p and 9p are more frequently observed in iGC, 8p and 12q gains are associated with dGC (12, 13), arguing that iGC and dGC may represent molecularly distinct entities. Furthermore, ERBB2/HER2 amplifications are observed in iGC but not dGCs, raising the possibility that only the former may be candidates for Trastuzumab (anti-HER2) therapy (14). Identifying additional genetic and genomic aberrations specifically associated with iGC or dGC might further elucidate the mechanistic basis of their morphologic differences, and identify potential avenues for iGC or dGC-specific treatments.

Recently, high-resolution aCGH platforms have allowed detection of genomic aberrations that are <40 kb (15). In this report, we used high-resolution aCGH to analyze a panel of GC cell lines and discovered a focal high level amplification at Chr 6p11p12 in Hs746T cells that has not been previously described. We identified RAB23 as an amplified and overexpressed gene located in this region, and confirmed RAB23 amplifications in primary tumors. We provide functional evidence that RAB23 regulates cell invasion in GC, and in two independent patient cohorts, RAB23 overexpression at both the RNA and protein level was strongly associated with dGC, providing further evidence that dGCs and iGCs likely develop via different molecular pathways (16, 17). Taken collectively, our study shows that targeting focal chromosomal amplifications using several different high-resolution techniques can lead to the rapid identification of candidate genes relevant to GC. Such strategies might also be extended to other cancer types.

Materials and Methods

Cell culture. GC cell lines were purchased from the American Type Culture Collection and cultured in 90% DMEM (high glucose) + 10% fetal bovine serum (FBS) for Hs746T; 90% RPMI + 10% FBS for AGS, NCI-N87, SNU-1, SNU-5, and SNU-16; and 80% RPMI + 20% FBS for KATOIII at 5% CO\textsubscript{2}, 37°C.

BAC array-CGH. Genomic DNA was extracted using a Qiagen extraction kit. Two micrograms of cell line and reference genomic DNA (unrelated...
pooled male lymphocyte DNA from healthy donors) were labeled with Cy3-dCTP or Cy5-dCTP, respectively, (Amersham Biosciences) and hybridized onto microarrays containing 32,000 DOP-PCR–amplified BAC clones covering the whole human genome (Children’s Hospital Oakland Research Institute, BAC PAC resources; ref. 15). Hybridizations were performed using a MAUI hybridization station, and scanned using a GenePix 400B scanner (Axon Instruments). Raw images were analyzed using GenePix Pro 4.0 software.

100K single-nucleotide polymorphism array. Genomic DNAs were hybridized onto Affymetrix GeneChip Human Mapping 50K XbaI arrays and 50K HindIII arrays according to the manufacturer’s instructions. Raw image files were analyzed using Dchip Software.6 The microarray data in this study has been deposited in the Gene Expression Omnibus database under accession number GSE10611.

Gene expression profiling. RNA was extracted using Trizol reagent (Invitrogen) and processed for Affymetrix GeneChip hybridizations on U133A Genechips according to the manufacturer’s instructions. Gene expression data were quality controlled by GeneData Refiner.7 Gene expression data from individual arrays were normalized by median centering around 500 expression units. Two or three experimental replicates were performed for each line (18).

Fluorescence in situ hybridization analysis. Single-cell nuclear suspensions were prepared using standard interphase fluorescence in situ hybridisation analysis (FISH) protocols. BACs specific to 6p11p12 (RP11-6H11011; CHORI) were labeled with Texas red–conjugated dUTP and mixed with FITC–labeled centromeric 6 probes (D6Z1; American Type Culture Collection). Up to 100 interphase nuclei and metaphases were assessed, and fluorescent images were analyzed using FISHview (Applied Spectral Imaging GmbH). For primary GCs, imprints from 10 primary GCs and matched normal gastric mucosa from fresh resection specimens were placed on glass slides. Probes for the primary tumor experiments were labeled with spectrum orange (D62Z1) or FITC (RP11-6H11011).

Real-time genomic PCR. Quantitative real-time PCR was performed on a LightCycler (Roche) using SYBR Green PCR Master Mix (Roche) and primers specific to the RAB23 and β-ACTIN (ACTB) genomic locus: RAB23_fwd, 5′-AGGCAAGACTCCGTCTCTCAA; RAB23_rev, 5′-CACCCCC-TAAGGTACCAGCATG; ACTB_fwd, 5′-GTGCGATCATCAGGAAACTC; ACTB_rev, 5′-AGCTAGCCAGAAGAACAC. Quantification of genomic copy number was based on standard curves, and each qPCR was performed either in duplicate or triplicate. For both cell lines and primary tumors, RAB23 gene copy numbers were compared relative to the ACTB genomic control within the same sample. For the cell line analysis, normal diploid DNAs were not included because the main purpose of this experiment was to compare RAB23 gene copy number levels across the lines. In contrast, normal diploid DNAs were used in the analysis of primary tumor samples to show increased RAB23 gene copy number levels in tumors compared to tumors with normal tissues.

Semiquantitative reverse transcription-PCR. RNA was reverse transcribed into cDNA using superscript II Reverse transcriptase (Invitrogen) and oligo-dT (T18) primers (Research Biolabs). Semiquantitative reverse transcription-PCR (RT-PCR) was performed using RAB23 and β-ACTIN specific primers (Research Biolabs): RAB23_fwd, 5′-GGTATCCCTAATGTTGGAGA; RAB23_rev, 5′-ATGACAAGTGGTAGGATTTT; ACTB_fwd, 5′-CGGAAAATCTTGCTGCCATTT; ACTB_rev, 5′-TGATCTCCTCTTGCATCTTGGGG.

Western blotting. Cell pellets were lysed in modified radioimmunoprecipitation assay lysis buffer with a cocktail of protease inhibitors (Roche). For each cell line, 30 μg of protein were separated on a 15% SDS polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane. The blots were probed with anti-RAB23 (1:1,000) or anti-β-ACTIN (1:1,000 Sigma) antibodies, incubated with horseradish peroxidase–conjugated secondary antibodies, and visualized using a chemiluminescence detection reagent (Bio-Rad).

siRNA and cDNA transfection. Hs746T and AGS cells were seeded into a 24-well plate at a density of 1.8 × 103 cells/mL and incubated for 24 h before transfection with either negative control siRNA or specific RAB23 siRNAs (200 nmol/L, SMART pool or individual RAB23 siRNAs; Dharmacon) by Oligofectamine (Invitrogen) in OptiMEM Medium (Invitrogen). The ‘negative control’ siRNA was a scrambled siRNA containing a random nucleotide sequence. For RAB23 overexpression, a full-length RAB23 cDNA was cloned into the pCI-Neo mammalian expression vector (Promega) and transfected into AGS cells using Oligofectamine reagent (Invitrogen). After 72 h of siRNA treatment, relative cell proliferation was determined by measuring absorbance using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche). Each assay was carried out in triplicate, and normalized relative to control-transfected cells.

Invasion and migration assays. Cell invasion assays were performed using Biocoat Matrigel invasion chambers with 8-μm pore filter inserts (BD Bioscience). The 8-μm pore size was selected for these experiments as this size has been used in other published studies (19–23). Forty-eight hours after transfection, Hs746T or AGS cells were trypsinized and transferred to the upper Matrigel chamber in 500 μL of serum-free medium containing 1 × 105 cells (5 × 104 for AGS cells) and incubated for 44 h. The 44-h time point was selected based on the invasive intrinsic rate of Hs746T cells, which is different between different cell lines. Migrating cells were counted using light microscopy. In the figures, we have inserted red arrows to highlight representative cells invading through the transwell, which should be distinguished from the 8-μm pores of the transwell material (small round circles). Each assay was performed in triplicate, and the results were averaged over three independent experiments. For cell migration assays, Hs746T cells were transfected with control or RAB23 siRNAs and seeded into a 6-well plate to reach 100% confluency at the time of maximum RAB23 knockdown. A “scratch” wound was inflicted across each well using a 200-μL pipette tip, and the rate of cells migrating into the scratch was observed. Pictures of the wound were taken at 24- and 48-h intervals, and cell migration distances across the wound were measured. Six parallel experiments were performed in a total of 24 wells.

Clinical samples. We tested three different patients cohorts, and the type of experiment performed on each cohort was based on the availability of genomic DNA, gene expression information, clinical data, or tissue microarrays (TMA).

Singapore: Anonymized primary human gastric tumors were obtained from the National Cancer Centre Singapore tissue repository with approval from the local Ethics Committee and signed patient informed consent. Tumor content in all samples was confirmed to be >50% by frozen sections. Genomic DNA from pooled healthy female and male blood lymphocytes were used as normalization controls.

Hong Kong: Gene expression data for 90 GC patients and 28 normal gastric tissues were obtained from genome-www.stanford.edu.8 United Kingdom (Leeds): 306 patients receiving surgical resections for gastric adenocarcinoma at the Academic Department of Surgery, Leeds General Infirmary, United Kingdom, between 1981 and 2000 were studied. One hundred ninety-one (62.4%) were male and 115 (37.6%) were female, with a median age of 72.1 y, ranging from ages 36 to 96 y. Additional clinicopathologic variables, including tumor-node-metastasis classification (22), tumor differentiation grade according to WHO classification (23), tumor stage, and histologic subtype according to Lauren classification (10) are provided in the Supplementary Data. This study was performed with the approval of the Leeds (West) Research Ethics Committee (LREC No C061/122).

TMAs and immunohistochemistry. Cell lines were fixed and embedded into paraffin, and used to construct a cell line array using a Beecher microarrayer (24). TMAs were constructed from routine formalin-fixed, paraffin-embedded GC tissue and matched normal mucosa (if available; ref. 25). Three cores were taken from tumors with high tumor cell density, and six cores from tumors with low tumor cell density (<30% tumor cells

http://bioscan1.harvard.edu/complab/dchip/ 6
http://www.genedata.com 7

Each TMA also contained several non-gastric tissue samples to assess sensitivity and specificity of the immunohistochemical staining. RAB23 primary antibodies were diluted 1:1,000 in antibody diluent (Zymed Laboratories), and biotinylated anti-rabbit (ready-made DAKO ChemMate Bottle A; DAKO Cytomation) was used as a secondary antibody. Further immunohistochemical details are provided in the Supplementary Data. The intensity of the RAB23 immunohistochemical reaction was scored as negative, weak, moderate, or strong. To avoid false-negative cases, a tumor was only categorized as "negative" if positive staining was present in non-malignant cells of the same core or any other core of the same case. In preliminary work, we found that RAB23 was expressed in a cell membrane–associated pattern in all normal gastric epithelial cells but in a predominantly cytoplasmic pattern in tumor cells (see Results and the Supporting Data). In the absence of normal gastric epithelial cells, positively stained lymphocytes or smooth muscle cells were used as internal controls. Cores without positive internal controls were excluded from the statistical analyses, resulting in the overall number of informative cases being 306.

**Statistical analyses.** Statistical analyses were done using SPSS 12.0.1 for Windows (SPSS, Inc.). For the analyses of RAB23 expression, comparisons for the different staining intensity categories were performed using either the Mann-Whitney test (for two groups, negative/weak versus moderate/strong) or the Kruskal-Wallis test (for more than two groups).

**Results**

High resolution aCGH identifies a focal chromosomal amplification at 6p11p12 in GC. To identify regions of chromosomal amplification in GC, we interrogated seven GC cell lines using high-resolution aCGH on two different platforms: a BAC-microarray platform comprising 32,000 BACs providing tiling resolution coverage of the whole human genome (15), and an oligonucleotide microarray platform containing probes representing 100,000 single-nucleotide polymorphism (SNP)s. We confirmed several previously described amplicons in this cell line panel, such as c-Myc amplification at chromosome 8q24 in SNU-16 cells, ERBB2 amplification at chromosome 17q21 in NCI-N87 cells, and c-Met amplification at chromosome 7q31 in SNU-5 cells ( Supplementary Fig. S1). We also discovered a novel high-level focal amplification at chromosomal region 11q22 in YCC3 and YCC7 cells (Supplementary Fig. S1), containing the genes BIRC2 and YAP1, recently identified as coamplified genes with cooperative effects in liver cancer (26). Taken collectively, these results support the validity and robustness of the aCGH data.

We identified an amplified region at Chr 6p11p12 at genomic position 56 Mb (genome build National Center for Biotechnology Information 36.1) in Hs746T cells (Fig. 1A). The enhanced resolution of our aCGH platform allowed us to narrow down the amplicon region to 3 Mb, compared with the 5.8 Mb obtained by conventional CGH (data not shown). The presence of this 6p11p12 amplification was confirmed by 100K SNP arrays (Fig. 1B and C, representing chromosomal and close-in views, respectively). Excellent concordance was seen between the BAC and SNP arrays (Fig. 1D), indicating that the same genomic aberration can be detected by using different high-resolution array technologies.

Figure 1. Identification of the chromosome 6p amplicon. A, aCGH profiles of chromosome 6 in GC cell lines. Y-axis, log2-transformed LOWESS smoothened values averaged over a dye-swap replicate. X-axis, the physical genomic location along chromosome 6. Black arrow, the region of interest. B, SNP array profiles of chromosome 6. Top red rows, inferred copy number for samples (median smoothing, window width is nine). Gray box on the bottom, the value range from 0 to 4 copies; red line, a normal copy number of 2. Blue curve in the gray box, the copy number of the Hs746T cell line. C, close-up views of inferred copy number for the amplified locus by SNP array (median smoothing, window width is nine). D, zoom-in diagrams for aCGH and SNP array profiles. Smoothened and unsmoothened data from replicates (colored dots) are shown for the cell line Hs746T. Red bars, peak region of amplification.
**RAB23**, a candidate 6p11p12 amplicon gene, is amplified and overexpressed in Hs746T cells. By integrating the BAC and SNP array results, we localized the peak of the 6p11p12 amplification to 57.1 and 57.4 Mb (Fig. 1D, red bar). Two candidate genes whose genomic coordinates were entirely encompassed within the amplification peak were identified, BCL2-associated genes whose genomic coordinates were entirely encompassed within the amplification peak were identified, BCL2-associated athanogene 2 (BAG2; Chr6: 57,145,293-57,157,694), and RAB23 (Chr6: 57,161,541-57,194,216), a small GTPase belonging to the Ras superfamily (Fig. 2A). Although BAG2 and RAB23 lie in extremely close proximity to one another, being separated only by ~4 kb, the two genes are transcribed in opposite directions, indicating that they possess distinct promoters. Besides BAG2 and RAB23, two other genes were partially covered by the amplification peak—ZNF451, a zinc finger protein, and PRIM2A, a large primase subunit involved in DNA replication. DST, a member of the plakin protein family found at adhesion junctions, was located close to this region but fell outside the predicted amplification peak.

To investigate if genes within the 6p11p12 amplicon might exhibit "copy number–driven expression," we analyzed gene expression values for these candidate genes from a gene expression profile database of GC cell lines (18). RAB23 was overexpressed in Hs746T by >4.5-fold relative to the median expression of all 7 GC cell lines (Supplementary Fig. S2). Other 6p11p12 genes involved in the predicted region of amplification were also overexpressed but to a lesser extent than RAB23. These include BAG2, followed by PRIM2A, ZNF451, and DST. Notably, the expression levels of other 6p11p12 genes outside the amplified region (e.g., HCRTR2, BMP5, and KHDNBS2) were not significantly different between Hs746T and the other cell lines (Supplementary Fig. S2), supporting the notion that the elevated expression levels of genes within this region in Hs746T cells is likely due to DNA amplification. The relatively high level of RAB23 overexpression motivated us to further investigate the potential role of this gene in GC.

**Validation of RAB23 overexpression—gene copy number, mRNA, and protein expression.** To confirm that the 6p11p12 region was indeed amplified in Hs746T cells, we conducted FISH experiments using chr6p11p12-specific probes (RP11-641O11) and a reference centromere 6 probe (D6Z1; Fig. 2B). In SNU-1 cells that did not show a 6p11p12 amplification by aCGH, we consistently observed a diploid ratio of 6p11p12 to centromere 6 signals (Fig. 2B). In contrast, in Hs746T cells, the modal number of 6p11p12 signals was 8, whereas the modal centromere 6 number was 5, indicating that the 6p11p12 region is indeed amplified relative to centromere 6. In Hs746T cells, the 6p11p12 signals localized to multiple scattered areas across the cell nucleus in contrast to being colocalized with the centromere 6 probe as in SNU-1 cells (Fig. 2B), suggesting that this region has translocated to multiple regions in different chromosomes.

To confirm RAB23 amplification at the single gene resolution, we quantified RAB23 gene copy numbers in these lines by genomic qPCR. Hs746T cells exhibited a four to five times increased gene copy number of RAB23 relative to a β-actin (ACTB) control gene (Fig. 2C). Semiquantitative RT-PCR showed that RAB23 (Fig. 2D) was significantly overexpressed in Hs746T cells compared with other cell lines confirming the genome-wide Affymetrix expression array results. Furthermore, Western immunoblotting using RAB23 antibodies showed that RAB23 was overexpressed in Hs746T cells compared with other cell lines (Fig. 2D). Interestingly, the immunoblotting results also revealed a considerable level of heterogeneity in RAB23 protein expression in the other lines.

RAB23 protein was also expressed in SNU-1 and AGS cells albeit at a lower level than in Hs746T cells; SNU-16 cells had no detectable RAB23 expression; and NCI-N87, KATO III, and SNU-5 cells had very weak RAB23 expression. These findings suggest that in addition to gene amplification, RAB23 expression in some cell lines may be modulated by other mechanisms.
A potential cell invasion role for RAB23 in amplified and nonamplified cell lines. To investigate the potential function of RAB23, we performed siRNA-mediated knockdown experiments. Using a pooled set of siRNAs targeting RAB23, we successfully silenced RAB23 at the mRNA and protein level in RAB23-amplified Hs746T cells (Supplementary Fig. S3; Fig. 3A). We also included in these experiments siRNAs targeting BAG2 (Supplementary Fig. S3). We found that there was no significant difference in cell proliferation capacity between control cells, RAB23-, BAG2-, and RAB23/BAG2-silenced cells (Supplementary Fig. S4). However, in a Matrigel invasion assay, both RAB23-silenced and RAB23/BAG2–double silenced Hs746T cells exhibited a decreased level of cell invasion compared with control cells (Fig. 3A and B; \( P = 0.01 \) and 0.005 for RAB23 siRNA and RAB23/BAG2 siRNA, respectively), suggesting a potential role for RAB23 in cell motility and invasion. In contrast, BAG2 silencing alone did not significantly affect cellular invasion (Fig. 3B).

To rule out potential off-target effects of the RAB23 pooled siRNA, we repeated the Matrigel invasion assays using two independent siRNAs (siRNA-5 and siRNA-6) targeting distinct parts of the RAB23 gene (Supplementary Fig. S3). The efficacy of the single siRNA treatments were confirmed by Western blotting (Supplementary Fig. S3). Similar to the pooled siRNA experiments, treating Hs746T cells with either RAB23 siRNA-5 or RAB23 siRNA-6 also resulted in a significant reduction of invasion (Supplementary Fig. S4; \( P = 0.0053 \) and 0.0011). These results indicate that the effects of the RAB23 siRNA treatment are unlikely to be due to an off-target effect. To test if the trends seen in the Matrigel experiments could also be observed in an independent assay, we performed cell migration “wound healing” assays (see Materials and Methods). Treatment of Hs746T cells with either RAB23 siRNA-5 or RAB23 siRNA-6 both resulted in a significant inhibition of cell migration (Supplementary Fig. S4; Fig. 3C; \( P = 0.0097 \) and 0.003 for RAB23 siRNA-5 and siRNA-6, respectively). These results support a functional role for RAB23 as an invasion mediator in GC.

We then investigated if the invasion mediator effects of RAB23 might also be observed in other lines. No effects of RAB23 siRNA were observed in non-RAB23–expressing cell lines such as YC3 and YCC10 (Supplementary Fig. S3; data not shown). In AGS cells, which express RAB23 but do not carry RAB23 amplifications (Supplementary Fig. S3; Fig. 2D), RAB23 knockdown resulted in a significant reduction of invasiveness similar to Hs746T cells (Supplementary Fig. S4; Fig. 3D; \( P = 0.028 \)). To extend this finding, we asked if RAB23 overexpression might prove sufficient to induce invasiveness in AGS cells. Using a RAB23 cDNA expression construct, we found that RAB23 overexpression resulted in a significant enhancement of invasion (Fig. 3D; \( P = 0.00072 \)). Taken collectively, these results suggest that RAB23 expression may influence the invasive capacity of a variety of different GC cells.

RAB23 expression is significantly associated with diffuse-type GC in multiple patient cohorts. To investigate RAB23 in primary cancers, we performed FISH on primary gastric tumors. We detected RAB23 amplification in 2 of 10 primary GCs (Fig. 4A). Of the remaining eight, five tumors exhibited a normal number of
RAB23 and chromosome 6 signals, two displayed concomitantly increased numbers of RAB23 and chromosome-specific signals suggesting gross aneuploidy, and one tumor exhibited increased chromosome 6 signals compared with RAB23. To extend these findings to a larger sample series, we used genomic qPCR to quantify RAB23 gene copy number in 70 primary GC samples from Singapore. We first measured RAB23 copy numbers relative to an ACTB genomic control in 4 diploid reference genomic DNAs and found a qPCR mean of 1.1-fold with a SD of 0.1—thus, measurements above 1.3-fold are likely to represent true deviations from normal DNA copy number at 95% confidence. Under these conditions, we identified 9 tumors (13%) exhibiting potential RAB23 expression gains with RAB23/ACTB ratios of >1.25. Of these, 3 to 5 tumors likely harbor RAB23 amplifications with RAB23/β-actin ratios of ≥1.5 (Fig. 4B). Taken collectively, these results show that RAB23 amplifications can indeed occur in primary GC.

To compare RAB23 gene expression between normal gastric tissues and cancers, we performed RAB23 quantitative RT-PCR on nine normal gastric tissues and 15 gastric tumors from Singapore. Using a cutoff threshold of >2-fold expression in cancers compared with the median RAB23 expression level in normal gastric tissues, 7 of 15 tumors (46%) overexpressed RAB23 (Supplementary Fig. S5). To extend these findings from the relatively small Singapore data set to a larger series with known histopathologic and clinical variables, we investigated a publicly available data set containing the gene expression profiles of 90 GCs from Hong Kong (27). Using the same threshold cutoff as the quantitative RT-PCR analysis (>2-fold greater expression in tumors compared with normal tissues), 35% of GCs in the Hong Kong cohort overexpressed RAB23 at the transcript level (Fig. 4C), a percentage comparable with the Singapore samples. Notably, the observation that more cancers overexpress RAB23 than show RAB23 gene amplification is consistent with the results we obtained from GC cell lines. No significant associations were found between RAB23 expression and tumor site or tumor stage (Supplementary Table S1). However, RAB23 expression level was significantly associated with histologic tumor type, with dGCs

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**Figure 4.** RAB23 gene, transcript, and protein expression in primary tumors. A, FISH analysis of primary tumors. Gastric tumor imprints were probed with a RAB23 6p11 probe, RP11-641011 (green), and a D6Z1 centromeric 6 probe (red). Left, gastric tumor cells exhibiting 6p11 amplification (multiple green signals) with a normal set of centromere 6 signals (two copies). Right, normal gastric epithelial cells from the same patient, exhibiting two copies of both RAB23 and centromere 6. 4,6-diamidino-2-phenylindole was used to counterstain the nucleus. B, genomic qPCR of RAB23 gene in 70 GC samples (Singapore [SG] cohort). Red columns, tumors exhibiting a RAB23/ACTB ratio of >1.25; dotted line, a threshold RAB23/ACTB cutoff of >1.5. Three to five tumors meet this latter threshold. C, gene expression profiles of RAB23 gene in 90 GC samples (Hong Kong [HK] cohort; ref. 27). Dotted line, RAB23 gene expression that is >2-fold above than the mean RAB23 expression level in normal gastric tissues. Red columns; tumors that pass this criterion. D, RAB23 immunohistochemistry in United Kingdom cohort. Hs746T, strong diffuse cytoplasmic immunoreactivity; KATO III, weak cytoplasmic immunoreactivity prominently in intracytoplasmic vesicle-like structure and cell membrane; dGC, strong diffuse cytoplasmic immunoreactivity of RAB23 in dGC; iGC, weak to no RAB23 immunoreactivity in iGC. Bars, 40 μm.
exhibiting significantly higher expression levels of RAB23 compared with iGCs (Spearson $\chi^2$, $P = 0.01$).

To validate the association between RAB23 expression and dGC at the protein level, we performed immunohistochemistry in a third independent cohort of >300 GC patients from the United Kingdom. First, we used cell lines to confirm the specificity and sensitivity of the RAB23 antibody in formalin-fixed paraffin-embedded material—similar to the immunoblotting data, different cell lines expressed different levels of RAB23, with RAB23 staining being strongest in Hs746T, and weakest in KatOII cells (Fig. 4D). In these lines, RAB23 was also predominantly located in cytoplasmic dots suggestive of being located in vesicles and was sometimes observed at the cell membrane (Fig. 4D). Second, RAB23 IHC was informative in 306 primary gastric adenocarcinomas and normal gastric mucosa. One hundred seventy-seven (58%) GCs showed moderate to strong immunoreactivity, and 129 (42%) GCs showed weak or no immunoreactivity for RAB23. Although normal gastric epithelial cells showed a membranous staining pattern with very little cytoplasmic staining (Supplementary Fig. S6), RAB23 in tumors was most commonly seen in a cytoplasmic “vesicular” and sometimes cytoplasmic diffuse pattern similar to the pattern in cell lines (Fig. 4D). Consistent with mRNA transcription findings in the Hong Kong data set, dGCs in the UK data set showed significantly higher RAB23 immunoreactivity compared with iGC ($P = 0.003$; Supplementary Table S2; Fig. 4D). No association of RAB23 expression was found with gender, depth of tumor invasion (pT), lymph node status (pN), tumor stage, and grade of differentiation (Supplementary Table S1). These results, observed at both the gene expression and protein level in two independent patient cohorts (Hong Kong and the United Kingdom), confirm that RAB23 expression is significantly and consistently associated with dGC.

Discussion

In this study, we used integrative genomics to identify novel genes involved in GC. By interrogating a panel of GC cell lines with two different high-resolution aCGH platforms and gene expression profiling, we identified and subsequently validated RAB23 as an amplified and overexpressed gene in Hs746T cells. Functionally, siRNA-mediated knockdown of RAB23 inhibited the invasive capacity of both RAB23-amplified (Hs746T) and nonamplified (AGS) cells, suggesting that RAB23 may play a role in cancer cell invasion. We confirmed the presence of RAB23 amplifications in primary tumors, and in two independent cohorts from Hong Kong and the United Kingdom, RAB23 transcript and RAB23 protein expression was associated with tumor morphology (i.e., dGC).

Our experimental approach was specifically targeted toward high-level focal genomic amplifications, rather than the more commonly observed large genomic aberrations in primary tumors. Compared with lower resolution technologies, the boundaries and central peaks of these focal amplifications are more robustly detected using high-resolution aCGH platforms due to their higher level of probe coverage. Indeed, similar strategies targeting focal high-level amplifications have also been informative in recent studies of liver (26) and oral cancer (28). One potential disadvantage of this strategy is that such focal amplifications are usually rarer compared with broad amplifications and, thus, might be easily missed if only small numbers of cell lines or samples are evaluated. This was observed in our study, where the 6p11p12 region was amplified in only one of the seven GC cell lines. However, we have subsequently observed amplification of this region in an expanded GC cell line panel (Supplementary Fig. S7). Furthermore, it should be remembered that other cellular mechanisms besides gene amplification may also cause overexpression of these genes. This was confirmed in our study, where RAB23 protein expression was also discovered in several non-RAB23–amplified cell lines (AGS and SNU-1; Fig. 2D), and in primary GCs, a larger fraction of tumors overexpressed RAB23 at the transcript level compared with the fraction with RAB23 gene amplifications (Fig. 4A and B versus C). This apparent discordance between RAB23 gene and protein is unlikely to be specific to RAB23—it is well-established that there exists an imperfect correlation between gene and protein expression (29, 30). This may be due to the activity of additional as-yet-unidentified regulatory mechanisms that differentially modulate transcript versus protein levels. However, this observation does not detract from our main conclusion that identifying genes under focal amplifications in cell lines, with subsequent validation in primary tumors, may prove a useful general strategy to identify new cancer related genes.

RAB23 is located at chromosome position 6p11p12, and chromosome 6p amplifications have been previously reported in GC using conventional CGH (2, 8). However, although RAB23 was the most overexpressed candidate gene in this 6p region, it is possible that other chromosome 6p genes, particularly those flanking RAB23, may also play a role in GC. Indeed, we also investigated the function of BAG2 as this gene is located in close proximity to RAB23. We found that reducing BAG2 expression by siRNA did not result in a significant reduction of cell proliferation, invasion, or apoptosis (Supplementary Fig. S2; Fig. 4; data not shown), suggesting that it may be a bystander gene. However, more work will be required to assess the potential role of other 6p11p12 genes in GC.

RAB23 is a member of the Rab GTPase family (31). Although our functional analysis suggests a role for RAB23 in cancer cell invasion, the exact cellular mechanism of RAB23 is currently unclear. RAB23 may play a role in facilitating vesicular transport, controlling endocytic progression to lysosomes (32). Interestingly, although RAB23 was originally reported to be predominantly expressed in the brain (32, 33), RAB23 has also recently been implicated in carcinogenesis. High RAB23 expression was reported in hepatocellular carcinoma patients and associated with tumor size (34). More pertinent to our study, another very recent report identified RAB23 as an up-regulated gene in nonmalignant diseased gastric tissues (e.g., atrophic gastritis with intestinal metaplasia) compared with normal gastric mucosa (35). Because atrophic gastritis may increase the risk of GC, these findings raise the possibility that RAB23 up-regulation may be involved at an early stage of gastric carcinogenesis. Furthermore, our discovery that RAB23 is both proinvasive and significantly associated with dGC is particularly intriguing because dGCs are phenotypically more invasive than iGCs. A preliminary survival analysis of patients expressing either high or low levels of RAB23 in tumors did not exhibit a statistically significant survival difference (data not shown). Nevertheless, our findings provide further support that iGCs and dGCs are likely to represent molecularly distinct entities, which may prove important for the development of subtype-specific targeted therapies.

What are the signaling pathways that RAB23 might regulate in GC? In the neural system, RAB23 may negatively regulate the sonic hedgehog signaling (SHH) pathway (36–38) by controlling the subcellular localization of essential SHH components (39, 40). In this regard, our finding that RAB23 is both amplified and overexpressed in GC may seem counterintuitive, as hyperactivation of SHH signaling has been shown to be tumorigenic in various cancer types such as small cell lung cancer (41), colorectal...
adenocarcinoma (42), medulloblastoma (43), and even GC itself (44, 45). However, SHH signaling has also been shown to be important in promoting parietal cell differentiation in the stomach (46, 47), and it is thus possible that a disruption of this differentiation pathway may be required for GC development. The potential role of RAB23 in influencing SHH signaling in GC deserves to be further examined. Alternatively, increased RAB23 expression (through gene amplification or other mechanisms) may influence cell migration and invasion in a manner independent of SHH signaling but still relevant to tumorogenesis.

In conclusion, we have successfully shown that an integrative genomic approach targeting focal high-level amplifications in cancer cell lines can lead to the identification of genes important in GC such as RAB23. Future work will need to focus on identifying additional focal amplifications using even higher resolution aCGH platforms (>1 million SNP) on more comprehensive cell line panels and larger patient cohorts. Furthermore, we plan to address if a molecular classification of GC as at least two separate diseases may facilitate our ability to identify novel treatment strategies for this deadly disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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