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Identification and functional analysis of 9p24 amplified genes in human breast cancer

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Abstract

Previously, our group identified a novel amplicon at chromosome 9p24 in human esophageal and breast cancers, and cloned the novel gene, *GASC1* (*gene amplified in squamous cell carcinoma 1*, also known as *JMJD2C*/*KDM4C*), from this amplicon. GASC1 is a histone demethylase involved in the deregulation of histone methylation in cancer cells. In the current study, we aimed to comprehensively characterize the genes in the 9p24 amplicon in human breast cancer. We performed extensive genomic analyses on a panel of cancer cell lines and narrowed the shortest region of overlap to approximately 2 Mb. Based on statistical analysis of copy number increase and overexpression, the 9p24 amplicon contains six candidate oncogenes. Among these, four genes (*GASC1 UHRF2*, *KIAA1432* and *C9orf123*) are overexpressed only in the context of gene amplification while two genes (*ERMP1* and *IL33*) are overexpressed independent of the copy number increase. We then focused our studies on the *UHRF2* gene, which has a potential involvement in both DNA methylation and histone modification. Knocking down UHRF2 expression inhibited the growth of breast cancer cells specifically with 9p24 amplification. Conversely, ectopic overexpression of UHRF2 in non-tumorigenic MCF10A cells promoted cell proliferation. Furthermore, we demonstrated that UHRF2 has the ability to suppress the expression of key cell-cycle inhibitors, such as $p16^{INK4a}$, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Taken together, our studies support the notion that the 9p24 amplicon contains multiple oncogenes that may integrate genetic and epigenetic codes and have important roles in human tumorigenesis.

Keywords

chromosome 9p24; GASC1; UHRF2; gene amplification

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Introduction

Cancer results from an accumulation of genetic and epigenetic aberrations. Genetic aberrations include chromosome number changes and translocations, gene amplifications, mutations and deletions (Vogelstein and Kinzler, 2004). Epigenetic abnormalities involve both altered patterns of histone modifications as well as losses or gains of specific DNA methylation (Esteller, 2007; Jones and Baylin, 2007). Genetic and epigenetic alterations in cancer cells interact directly and indirectly. For example, a genetic alteration in the gene encoding an 'epigenetic regulator' can lead to changes within the histone code and DNA methylation, which are subsequently involved in tumorigenesis in multiple tumor types. Identification and characterization of genetic and epigenetic aberrations, as well as their interconnections, will provide important insights into the pathogenesis of cancer.

Gene amplification, which can affect gene expression by increasing gene dosage, is a well-known oncogene-activating mechanism (Albertson *et al.*, 2003; Albertson, 2006). Canonical oncogenes, such as *ERBB2*, *CCND1* and *MYC*, have previously been identified as amplification targets linked to the development, progression or metastasis of human cancers, including breast, prostate, lung and other cancers (Albertson *et al.*, 2003; Vogelstein and Kinzler, 2004). Previously, mapping of the 9p24 amplicon in esophageal cancer cell lines led us to the positional cloning of the *gene amplified in squamous cell carcinoma 1* (*GASC1* also known as *JMJD2C*/*KDM4C*) gene. More recently, we identified *GASC1* as one of the amplified genes at the 9p24 region in breast cancer, particularly in basal-like subtypes. Our *in vitro* assays demonstrated that GASC1 can induce transformed phenotypes when overexpressed in immortalized, non-transformed mammary epithelial MCF10A cells (Liu *et al.*, 2009).

In the past, focal amplicons found in cancer specimens were considered to harbor a single driving oncogene, such as the *ERBB2* oncogene in the 17q12 amplicon (Fukushige *et al.*, 1986). However, recent extensive genomic analysis and functional studies provide evidence to suggest that common amplicons in cancer cells contain multiple oncogenes that can act independently or cooperatively in mediating neoplastic transformation. For example, we and several other laboratories have demonstrated that the 8p11-12 amplicon harbors several driving oncogenes with transforming function when the amplicon is present in breast cancers, particularly in luminal subtypes (Yang *et al.*, 2004, 2006, 2010; Garcia *et al.*, 2005; Gelsi-Boyer *et al.*, 2005; Pole *et al.*, 2006). In the current study, we aimed to comprehensively characterize the 9p24-amplified genes for potential roles in human breast cancer. Results obtained from our studies support the notion that the 9p24 amplicon contains multiple candidate genes, including *GASC1* and *ubiquitin-like with* plant homeodomain *and ring finger domains 2* (*UHRF2*), that may integrate genetic and epigenetic codes and thus have important roles in human tumorigenesis.

Results

High-resolution array comparative genomic hybridization (CGH) narrowed a focal chromosomal amplification at 9p24 in cancer

Previously, our group identified an amplicon at the 9p24 chromosomal region in human esophageal cancer and identified the novel oncogene *GASC1* from this amplicon (Yang *et al.*, 2000). Later studies showed a gain/amplification of the *GASC1* region in 7 of 50 breast cancer cell lines, including HCC1954, Colo824, SUM-149, HCC70, HCC38, HCC2157 and MDA-MB-436 (Neve *et al.*, 2006; Liu *et al.*, 2009). To further demonstrate that the 9p24 region is amplified in various tumor specimens, we searched the recently published array CGH database in a collection of 3131 copy-number profiles across multiple cancer types (Beroukhim *et al.*, 2010). Copy number increases at the 9p24 region mostly occurred in

small-cell lung, breast and esophageal squamous cancers. In 243 breast cancer samples, approximately 15% contained 9p24 gains, and 4.53% of cases had high-level amplification based on Genomic Identification of significant Targets in Cancer (GISTIC) analysis (Supplementary Figure S1) (Beroukhim *et al.*, 2010).

The frequent occurrence of the 9p24 amplicon in various human tumors underscores its importance in tumorigenesis. For the purpose of further characterization of the 9p24 amplicon, we carried out high-resolution array CGH (Agilent 244 K chip, Santa Clara, CA, USA) analysis of four cancer cell lines, including three breast cancer cell lines, Colo824, HCC1954 and HCC70, and one esophageal cancer cell line, KYSE150 (Shimada *et al.*, 1992; Yang *et al.*, 2000; Liu *et al.*, 2009). In our array CGH study, high-level copy number gain (amplification) was defined by a log2 ratio ≥1 and low-level copy number gain by a log2 ratio between 0.3 and 1. The 244 K array CGH confirmed our previous findings that all four cell lines contain 9p24 amplification, and provided the amplicon boundaries at high resolution (Figure 1a and Supplementary Table 1A) (Yang *et al.*, 2000; Liu *et al.*, 2009). Of note, we found that the centromeric boundary of the 9p24 amplicon in KYSE150 cells is located within the coding region of a *protein tyrosine phosphatase, receptor type, D* (*PTPRD*) gene, resulting in amplification of the C-terminal region, but deletion of the N-terminal region of the gene (Supplementary Table 1A and Supplementary Figure S2). The *PTPRD* gene spans approximately 2.3 Mb, from 8.30 to 10.60 Mb, and is represented by 201 probes in Agilent 244 k CGH arrays (Supplementary Table 1A). We validated our CGH results by real-time PCR using primers specific for the PTPRD's intron 7–exon 8 and intron 8–exon 9 sequences (Supplementary Figure S3). As shown in supplementary Figure S4, compared with the control cells that do not have 9p24 amplification, KYSE150 cells had an elevated copy number of *PTPRD* intron 8–exon 9, whereas the copy number of *PTPRD* intron 7–exon 8 in KYSE150 was lower than that of the control, implying that the amplification/deletion break point is located in this region. Interestingly, recent published genomic data indicated that the centromeric boundaries of the 9p24 gain/amplification region in basal-like primary breast tumor (~8.28 Mb), brain metastasis (~8.88 Mb) and xenograft samples (~7.78 Mb) are also adjacent to or located at PTPRD genome region (Supplementary Figure S5) (Ding *et al.*, 2010).

In order to define the minimal common region of gain/amplification, we also analyzed our previous 44 k array CGH data obtained from the SUM-149 breast cancer cell line (Supplementary Table 1B) (Liu *et al.*, 2009). Compared with HCC1954 and Colo824 cells, SUM-149 cells exhibited low-level copy number gain ($0.3 \leq \log_2$ ratio <1.0) at the 9p24 region. In agreement with this data, we demonstrated in our previous fluorescence *in situ* hybridization study that 10–14 copies of the GASC1 BAC probe were observed in the interphase nuclei of HCC1954 cells, while only 5–7 copies of the probe were observed in the SUM-149 cells (Liu *et al.*, 2009). Furthermore, our array CGH revealed the distal boundary of 9p24 gain in SUM-149 cells maps to 5.53–5.76-Mb site (Supplementary Table 1B). Combination of our array CGH data with that published by other groups allowed us to narrow down the commonly gained/amplified 9p24 region to approximately 2 Mb, from 5.53 to 7.78Mb (Figure 1b, Supplementary Table 1 and Supplementary Figure S5).

The 9p24 amplicon contains multiple candidate oncogenes

Accumulated evidence suggests that the common amplicons occurring in breast and other cancers contain multiple oncogenes that could have a role in cancer initiation and progression. As mentioned above, the shortest region of overlap of the 9p24 amplicon spans approximately 2 Mb, and excluding pseudogenes, contains 11 genes (Figure 1b and Table 1). We carried out real-time RT–PCR to measure the expression level of these genes in a panel of cancer cell lines with or without 9p24 amplification (Figure 2a and Table 1). We

then used Kendall's tau, a measure of association, to assess if the association between copy number and expression for each gene is statistically significant. Using $P = 0.01$ as a cut-off for a statistically significant association, we confirmed that *GASC1* is a target of the amplicon. In addition, we identified three new potential targets, *UHRF2, KIAA1432* and *C9orf123* (Table 1). In contrast, the elevated expression of two genes, *ERMP1* and *IL33*, is independent of their copy number status in human cancer cells (Figure 2a and Table 1). However, *ERMP1* and *IL23* are also potential oncogene candidates because of their frequent overexpression. We measured protein levels of GASC1 and UHRF2 by western blot analysis in a panel of breast cancer cell lines. These experiments demonstrate that Colo824, HCC1954, HCC70 and SUM-149 cells expressed higher levels of GASC1 and UHRF2 than breast cancer cell lines without gene amplification (Figure 2b). Thus, we propose that the 9p24 amplicon contains five candidate oncogenes in addition to *GASC1*, including *UHRF2, KIAA1432, C9orf123, ERMP1* and *IL33*, all of which could have a role in tumorigenesis.

UHRF2 gene amplification and overexpression promotes cell proliferation

The UHRF2 is a nuclear protein involved in cell-cycle regulation (Mori *et al.*, 2002; Bronner *et al*., 2007). We therefore sought to examine the biological effect of UHRF2 knockdown on the proliferation of breast cancer cells with 9p24 amplification. To perform knockdown experiments, we obtained two pGIPZ-UHRF2 short hair pin RNA (shRNA) expression constructs from OpenBiosystems (http://www.openbio systems.com/). In this pGIPZ vector, TurboGFP and shRNA are part of a single transcript allowing the visual marking of the shRNA-expressing cells. HCC1954 cells were transduced with the pGIPZ-UHRF2 shRNA, and a non-silencing shRNA lentivirus at a similar titer was used in parallel as the negative control. We selected cells with puromycin 48 h after infection. Pooled cell clones were monitored for TurboGFP expression by fluorescence microscopy. UHRF2 expression levels were measured by real-time RT–PCR, which revealed that the UHRF2-shRNA cell clones showed downregulation of UHRF2 expression to 30–45% of the level seen in the non-silencing shRNA-infected cell clones (Figure 3a). UHRF2 shRNA#2 more effectively knocked down expression than shRNA#1, and thus we used it in five cell lines: HCC1954 and HCC70 with UHRF2 gene amplification, SUM-52 and SUM-102 without the amplification as well as the non-tumorigenic MCF10A cells, which also lack the amplification. Subsequently, the effect of decreased UHRF2 expression on cell proliferation was examined. Knocking down UHRF2 inhibited the growth of HCC1954 and HCC70 cells by approximate 50%, but had only a minor effect on SUM-52, SUM-102 and MCF10A cells ($P<0.01$) (Figures 3b and c). The inhibition of HCC1954 cell growth by knockdown of UHRF2 was reproduced with the UHRF2 shRNA#1 (data no shown). Thus, UHRF2 knockdown has a more profound growth inhibition effect on cells with *UHRF2* gene amplification than in cells without the amplification.

The effects of UHRF2 on cell growth and transformation were further examined by ectopic overexpression in the non-tumorigenic MCF10A cells. Lentivirus carrying either the control vector or a UHRF2 expression construct was transduced into MCF10A cells and stable, independent clones were isolated. Western blot confirmed the overexpression of UHRF2 protein in these clones (Figure 4a). Compared with the control, MCF10A cells overexpressing UHRF2 grew more rapidly than control cells ($P<0.05$), and this growth advantage was reversed by UHRF2 shRNA (Figure 4b). However, MCF10A-UHRF2 cells retained the parental cells' characteristics of anchorage- and growth factor-dependent growth (data no shown). Taken together with the UHRF2 knockdown results, our data indicate that UHRF2 has a role in cell proliferation in breast cancer cells with the 9p24 amplification.

UHRF2 mediates tumor suppressor gene inactivation in breast cancer

UHRF family members, including UHRF1 and UHRF2, are multi-domain proteins that participate in methylation-dependent transcriptional regulation (Bronner *et al.*, 2007; Unoki *et al.*, 2009; Rottach *et al.*, 2010). Recent studies revealed that UHRF1 functions as a transcriptional co-repressor and participates in transcriptional regulation of $p21^{Waf1/Cip1}$ by recruitment of DNA and histone methyltransferases (Kim *et al.*, 2009; Unoki *et al.*, 2009). Knocking down UHRF2 affects the expression level of $p21^{Waf1/Cip1}$ in lung cancer cells (He *et al.*, 2009). To determine whether UHRF2 affects $p21^{Waf1/Cip1}$ expression in human breast cancer cells, we examined $p21^{Waf1/Cip1}$ mRNA and protein levels after UHRF2 knockdown in HCC1954 cells. As shown in Figures 5a and b, UHRF2 knockdown resulted in increased expression of $p21^{Waf1/Cip1}$ at both the mRNA and protein levels. $p21^{Waf1/Cip1}$ is a well-known target gene of p53-mediated transcriptional regulation (el-Deiry *et al.*, 1993). However, HCC1954 cells harbor an inactivating mutation (Tyr163→Cys163) in the p53 gene (Sjoblom *et al.*, 2006). As expected, our western blot demonstrated that the expression level of p53 was not affected by UHRF2 knockdown in HCC1954 cells (Supplementary Figure S6), indicating that the increased expression of $p21^{Waf1/Cip1}$ in UHRF2 knockdown cells was achieved through a p53-independent pathway.

To determine whether UHRF2 affects the expression of other cell-cycle inhibitors and/or classical tumor suppressors, we examined the expression levels of $p16^{INK4a}$, $p27^{KIP1}$ and pRB after UHRF2 knockdown in HCC1954 cells. As shown in Figures 5a and b, when the expression of UHRF2 was decreased, there was a concomitant increase in the expression levels of $p16^{INK4a}$, $p27^{Kip1}$ and pRB. We further examined the expression levels of $p21^{Waf1/Cip1}$, $p16^{INK4a}$, $p27^{KIP1}$ and pRB in MCF10A-UHRF2 clones. As shown in Figure 5c, overexpression of UHRF2 in MCF10A cells led to reduced expression of $p21^{Waf1/Cip1}$, $p16^{INK4a}$ and $p27^{KIP1}$. However, the level of pRB protein was not affected in MCF10A-UHRF2 cells (Figure 5c). These data suggest that amplification and overexpression of UHRF2 suppresses the expression of tumor suppressor genes in cancer cells, which may explain its growth-promoting capability.

Discussion

Recent studies have demonstrated that regions of amplification such as 8p11-12, 11q13, 17q22-23 and 20q12-13 can be complex and frequently contain multiple genes that can work individually and/or in combination to influence the transformed phenotype in human cancer cells (Santarius *et al.*, 2010). Previous studies revealed the existence of 9p24 amplification in various tumor types (Italiano *et al.*, 2006; Han *et al.*, 2008; Vinatzer *et al.*, 2008; Liu *et al.*, 2009; Natrajan *et al.*, 2009; Northcott *et al.*, 2009). In human breast cancer, it had been determined that 9p24 amplification more frequently occurs in the basal-like subtype, which is clinically characterized as highly aggressive and is usually associated with a poor prognosis (Han *et al.*, 2008; Liu *et al.*, 2009). In the present studies, we extended our previous work on the 9p24 amplicon and examined 9p24 genes in a thorough and systemic way (Yang *et al.*, 2000; Liu *et al.*, 2009). Our array CGH analyses at a higher resolution enabled us to narrow the amplicon to approximately 2 Mb, which contains 11 genes. We identified four genes, *GASC1*, *UHRF2*, *KIAA1432* and *C9orf123*, that were overexpressed in association with copy number increase at the $P<0.01$ level (see Table 1). In addition, two genes, *ERMP1* and *IL33*, were found to be overexpressed in breast cancer cells both with and without copy number increases. Thus, like other focal amplicons found in cancer, the 9p24 amplicon also contains multiple candidate oncogenes.

Based on the known biological functions of the six candidate oncogenes, GASC1 and UHRF2 appear to have a role in the regulation of gene expression by acting as epigenetic regulators. The *KIAA1432* gene encodes a binding partner of a gap junction protein (GJA1,

also known CX43). The association with KIAA1423 protein is important for GJA1 to have a role as a gap junctional channel (Akiyama *et al.*, 2005). The *C9orf123* gene encodes a putative transmembrane protein, and its biological function is currently unknown. The ERMP1 is an endoplasmic reticulum-bound peptidase and required for normal ovarian histogenesis (Garcia-Rudaz *et al.*, 2007). As a cytokine, interleukin-33 may function as an alarm in that it is released upon endothelial or epithelial cell damage (Kurowska-Stolarska *et al.*, 2011). By contrast, the *PTPRD* gene, likely inactivated by partial deletion and/or rearrangement, is increasingly thought to be a tumor suppressor gene. Recent studies indicate that inactivation of *PTPRD* by gene deletion or mutation contributes to the pathogenesis of a wide range of human cancers, including colon, lung, glioblastoma and melanoma (Ostman *et al.*, 2006; Solomon *et al.*, 2008; Veeriah *et al.*, 2009; Kohno *et al.*, 2010; Giefing *et al.*, 2011). In breast cancer cells, it has been reported that *PTPRD* can also be inactivated at the transcriptional level by DNA hypermethylation (Chan *et al.*, 2008). Future investigations are required to more precisely address the role of each candidate gene in cancer development.

Using esophageal cancer lines, we originally identified and cloned the *GASC1* gene from an amplified region at 9p24 (Yang *et al.*, 2000). Based on the presence of a bipartite nuclear location sequence and two plant homeodomain fingers, we had initially predicted a role in transcriptional regulation for GASC1 (Yang *et al.*, 2000). Indeed, subsequent studies identified GASC1 as a member of the JMJD2 (jumonji domain containing 2), subfamily of jumonji genes that alter chromatin architecture through histone lysine demethylase activity (Katoh, 2004; Cloos *et al.*, 2006; Tsukada *et al.*, 2006; Whetstine *et al.*, 2006). Specifically, GASC1 can activate transcription by removing the repressive tri- and dimethylated histone H3 lysine 9 marks (H3K9me3/me2) at specific genomic loci (Chen *et al.*, 2006; Cloos *et al.*, 2006; Whetstine *et al.*, 2006; Klose and Zhang, 2007; Shi and Whetstine, 2007). We and several other laboratories showed that GASC1 regulates the expression of several classical oncogenes, including *MYC*, *NOTCH1*, *SOX2* and *MDM2* in normal and cancer cells (Loh *et al.*, 2007; Ishimura *et al.*, 2009; Liu *et al.*, 2009; Wang *et al.*, 2010). Importantly, stable overexpression of GASC1 in the non-tumorigenic breast cell line MCF10A induces transformed phenotypes, whereas knockdown in tumor cells inhibits proliferation, consistent with *GASC1* as a member of a new class of oncogenes that are involved in the deregulation of histone methylation in cancer cells.

A finding of particular interest from our current study is that the newly identified candidate *UHRF2* also has a potential involvement in methylation-dependent transcriptional regulation. UHRF2, and its close homolog UHRF1, contain similar functional domains. These domains include an ubiquitin-like domain, a plant homeodomain domain, a tudor domain, a SRA domain and a RING domain (Hopfner *et al.*, 2000; Mori *et al.*, 2002; Bronner *et al.*, 2007; Rottach *et al.*, 2010). Recent studies demonstrated that UHRF1 has the ability to bind hemi-methylated DNA and methylated H3K9 through its SRA domain and tudor domain, respectively (Bronner *et al.*, 2007; Qian *et al.*, 2008; Rottach *et al.*, 2010). UHRF1 can repress transcription of tumor suppressor genes including $p16^{INK4a}$ and $p21^{Waf1/Cip1}$ via recruitment of DNA methyltransferases (DNMT1 and DNMT3A/B), H3K9 methyltransferases (G9a), and HDAC1, interconnecting DNA methylation and histone modification pathways (Kim *et al.*, 2009; Unoki *et al.*, 2009). Interestingly, an unbiased proteomic screen for binding proteins to modified lysines on histone H3 also determined that UHRF2 interacts with dimethylated H3K9 peptide (Chan *et al.*, 2009). In the current study, we demonstrated that UHRF2 has the ability to repress transcription of key cell-cycle inhibitors and tumor suppressors, including $p16^{INK4a}$, p21Cip1 and $p27^{Kip1}$. Thus, we speculate that UHRF2 may have an oncogenic role by mediating tumor suppressor gene inactivation via both DNA methylation and histone modification pathways.

During the review of this manuscript, Rui *et al.* (2010) published their studies on 9p24 amplification in primary mediastinal B-cell lymphoma (PMBL) and Hodgkin lymphoma. They identified a 9p24 amplicon, which largely overlaps with the 9p24 amplicon described in this report. They revealed that within an approximately 3.5-Mb minimal common region of copy number gain, 10 genes (*JAK2*, *C9orf46*, *CD274*, *PDCD1LG2*, *KIAA1432*, *KIAA2026*, *RANBP6*, *UHRF2*, *GLDC* and *GASC1*) were upregulated in expression in association with gene amplification. Further, they demonstrated that two genes, *JAK2* and *GASC1*, cooperate to modify the epigenome of 9p24-amplified lymphomas, thereby promoting proliferation and survival. Their data and our studies share in common the observation that three genes, *GASC1*, *UHRF2* and *KIAA1432*, are upregulated via gene copy number gains, and that *GASC1* is an important gene for the proliferation and survival of cancer cells with 9p24 amplification (Liu *et al.*, 2009; Rui *et al.*, 2010). Notably, our array CGH and previous fluorescence *in situ* hybridization analysis found that JAK2 is not gained/amplified in KYSE150 esophageal cancer cells or SUM-149 breast cancer cells (Yang *et al.*, 2000; Liu *et al.*, 2009). Our new finding suggests that the epigenetic regulator UHRF2 likely contributes to cell proliferation in a subset of breast cancer with 9p24 amplification. It will be important to further investigate whether the two 9p24 co-amplified genes, *GASC1* and *UHRF2*, promote tumor growth co-operatively or independently.

Materials and methods

Cell culture

The culture of cancer cells KYSE150, Colo824, HCC70, HCC1954, SUM-44, SUM-52, SUM-149, SUM-190, SUM-225, and the immortalized non-tumorigenic MCF10A cells is described in the Supplementary Materials and methods.

Array CGH

Genomic array CGH experiments were done using the Agilent 244 K human genome CGH microarray chip (Agilent Technologies, Palo Alto, CA, USA) as described previously (Yang *et al.*, 2006). Briefly, for each array, female DNA (Promega, Madison, WI, USA) was used as a reference sample and labeled with Cy-3. The samples of interest were each labeled with Cy-5. Agilent's CGH Analytics software was used to calculate various measurement parameters, including log2 ratio of total integrated Cy-5 and Cy-3 intensities for each probe. Array data have been posted at the NCBI GEO database (GEO accession: GSE28989, GSM718287, GSM718288, GSM718289, GSM718290).

Real-time RT–PCR

Total RNAs were prepared from cells by using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and were converted into complementary DNAs with the qScript complementary DNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Primers were purchased from Invitrogen (Carlsbad, CA, USA). Real-time RT–PCR was performed using the iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

Lentivirus-mediated UHRF2 shRNA knockdown or overexpression

UHRF2 knockdown was achieved by using the Expression Arrest GIPZ lentiviral shRNAmir system (OpenBiosystems). The lentiviral expression construct expressing the *UHRF2* gene (pLenti-UHRF2-V5) was established as described previously (Yang *et al.*, 2006). Lentivirus was produced by transfecting 293FT cells with the combination of the lentiviral expression plasmid DNA and viral packaging mix (OpenBiosystems). Cells were infected with the virus by incubating with the mixture of growth medium and virus-containing supernatant (1:1 ratio), supplemented with polybrene at a final concentration of 5

μg/ml. An equal volume of fresh growth medium was added after 24 h and selection of stable cells was started after 48 h.

Examination of cell growth

Cell growth was assessed by using a Coulter counter or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). For MTT assay, cells were seeded in six-well plates at a density of 2×10^4 cells per well and allowed to attach overnight. At designated time points, thiazolyl blue tetrazolium bromide (Sigma-Aldrich, St Louis, MO, USA) was added to each well of cells (final 0.5 mg/ml) and incubated for 3–5 h at 37 °C. After removing the growth medium, dimethyl sulfoxide was added to solubilize the blue MTT-formazan product, and the samples were incubated for a further 30 min at room temperature. Absorbance of the solution was read at a test wavelength of 570 nm against a reference wavelength of 650 nm.

Immnuoblotting and antibodies

Whole cell lysates were prepared by scraping cells from the dishes into cold radioimmuno precipitation assay lysis buffer and sonicating for 10 s. After centrifugation at high speed in the cold, protein content was estimated with the Bradford method. A total of 20–100 μg of total cell lysate was resolved by SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Antibodies used in the study were as follows: anti-UHRF2 (Abcam ab28673, Cambridge, MA, USA), anti-GASC1 (Bethyl Laboratories A300-885A, Montgomery, TX, USA), anti- $p21^{Waf1/Cip1}$ (Cell Signaling 2947, Danvers, MA, USA), anti-p53 (Calbiochem Ab-2 OP09, Gibbstown, NJ, USA), anti-$p27^{Kip1}$ (Oncogene NA35, Cambridge, MA, USA), anti-$p16^{INK4a}$ (Oncogene NA29), anti-RB (Proteintech Group 10048-2-Ig, Chicago, IL, USA), anti-GAPDH (Cell Signaling 2118, Danvers, MA, USA) and anti-β-actin (Sigma-Aldrich A5441).

Statistical analysis

Kendall's tau was used to assess the statistical significance of the association between copy number and expression for each gene. Holm's step-down procedure was used to adjust significance level for the large number of estimates to reduce the likelihood of false positive results. We used $P = 0.01$ as a cut-off for a statistically significant association between copy number and expression. For analyzing the results of cell growth, a two-tailed independent Student's *t*-test was performed. A value of $P<0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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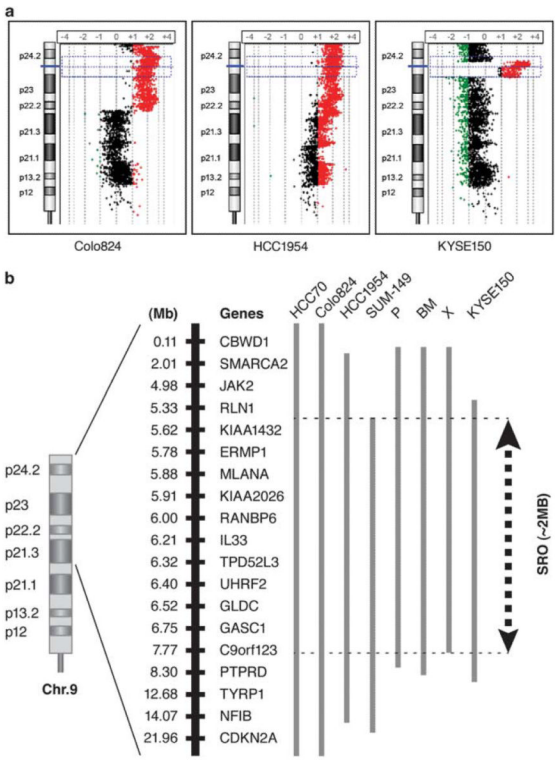

Figure 1.

Genomic analysis of the 9p24 region in human cancer cell lines. (**a**) Genome view of chromosome 9p analyzed on the Agilent oligonucleotide array (Agilent Technology) in Colo824, HCC1954 and KYSE150 cells. (**b**) Schematic representation of the 9p24-amplified region in four breast cancer cell lines (HCC70, HCC1954, Colo824 and SUM-149), one esophageal cancer cell line (KYSE150) and the recently published genomic data of basal-like primary breast tumor (P), brain metastasis (BM) and xenograft (X) samples (Ding *et al.*, 2010). Localization of the 9p21-24 genes is shown to the right of the chromosome 9 ideogram. The lines at far right represent the amplified region of each sample based on our array CGH data and Ding *et al.*'s published data. SRO, shortest region of overlap.

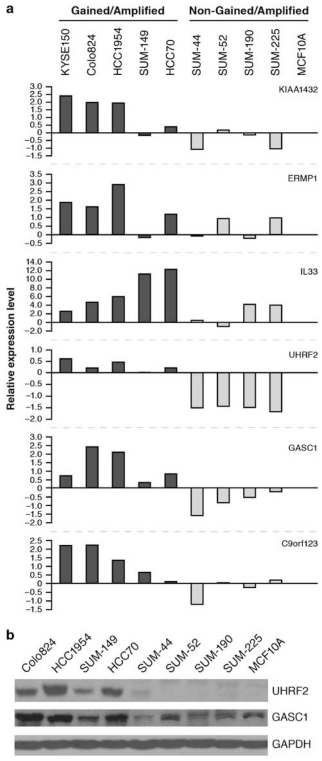

Figure 2.

(**a**) Expression level of six genes in the 9p24 amplicon. Gene expression was examined in cancer cells with 9p24 gain/amplification (KYSE150, Colo824, HCC1954, SUM-149 and HCC70) or without the gain/amplification (SUM-44, SUM-52, SUM-190 and SUM-225). mRNA expression levels in the MCF10A cells, an immortalized but non-tumorigenic breast epithelial cell line without 9p24 gain/amplification, were arbitrarily set as 0. Relative expression levels were shown as $\log_2$ values. (**b**) UHRF2 and GASC1 protein levels were analyzed by western blot in eight breast cancer cell lines with or without 9p24 amplification, as well as in MCF10A control cells.

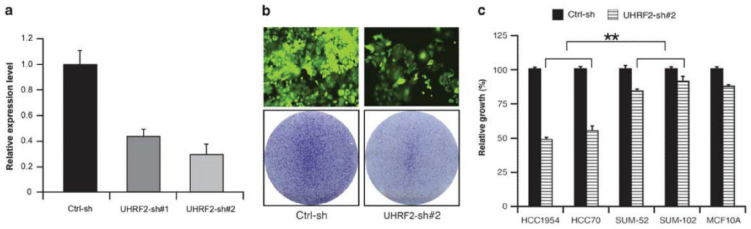

Figure 3.

Effect of UHRF2 knockdown on cancer cell growth. (**a**) Knockdown of UHRF2 mRNA in HCC1954 cells with two different shRNAs was confirmed by real-time RT–PCR. The real-time RT–PCR data were normalized with a GAPDH control and is shown as the mean±s.d. of triplicate determinations from two independent experiments. The baseline for the cells infected with control shRNA was arbitrarily set as 1. (**b**) Top panel shows TurboGFP images of HCC1954 cells after viral infection with control shRNA and UHRF2 shRNA#2. After seeding the same number of HCC1954 cells with or without UHRF2 knockdown, cells were stained with crystal violet at day 7 (bottom panel). (**c**) Relative cell growth after knocking down UHRF2 in five cell lines: HCC1954 and HCC70 with 9p24 amplification, SUM-52 and SUM-102 without the amplification as well as non-tumorigenic MCF10A cells. The same number of cells were seeded and allowed to grow for 7 days after attachment. Relative growth is shown as the mean±s.d. of triplicate determinations (**P<0.01).

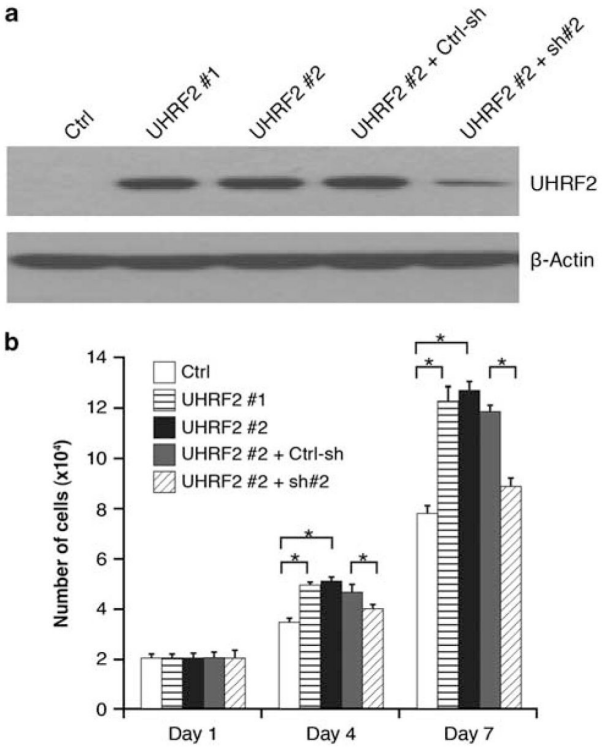

Figure 4.

(**a**) Stable overexpression of UHRF2 in MCF10A cells with the pLenti6/V5-UHRF2 construct (MCF10A-UHRF2). Overexpression of UHRF2 protein in two cell clones (UHRF2#1 and UHRF2#2), and knockdown of UHRF2 in clone #2 cells were confirmed by western blot. (**b**) Ectopic overexpression of UHRF2 confers a growth advantage to MCF10A cells, which can be reversed by UHRF2 shRNA (**P*<0.05).

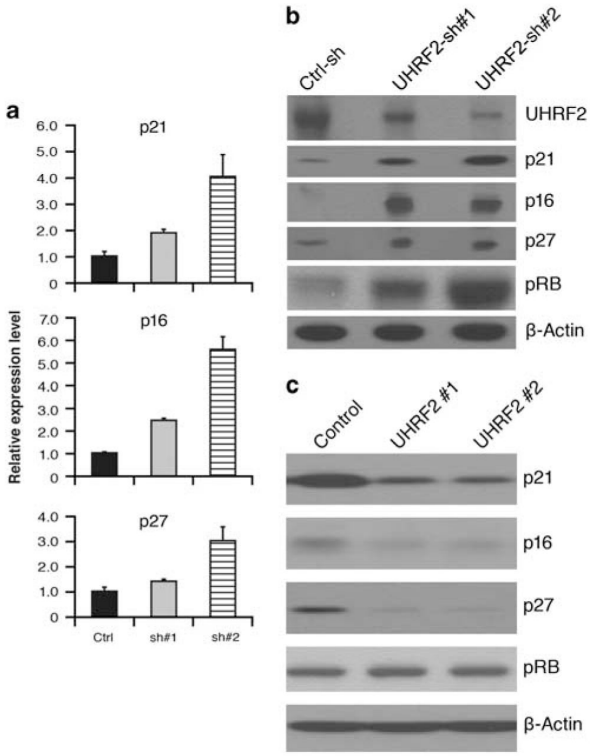

Figure 5.

UHRF2 influences expression of $p16^{INK4a}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and pRB. (**a**) mRNA levels of $p21^{Waf1/Cip1}$, $p16^{INK4a}$ and $p27^{Kip1}$ were examined by real-time RT–PCR after knocking down UHRF2 in HCC1954 cells. The baseline for the cells infected with control shRNA was arbitrarily set as 1. (**b**) Protein levels of $p21^{Waf1/Cip1}$, $p16^{INK4a}$, $p27^{Kip1}$ and pRB in HCC1954 cells stably expressing control shRNA, UHRF2 shRNA#1 or shRNA#2 were analyzed by western blot. The migration control for the hypophosporylated (p) form of RB protein is shown in Supplentmentary Figure S7. (**c**) Overexpression of UHRF2 in MCF10A cells results in reduced protein levels of $p16^{INK4a}$, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, but not of pRB as determined by western blot.

Table 1

Statistical analysis of association between copy number and expression of genes within the 9p24 amplicon

Gene	*Description*	*Kendall's tau*	P-*value*
KIAA1432	KIAA1432	0.78	<0.01
ERMP1	Endoplasmic reticulum metallopeptidase 1	0.67	0.02
MLANA	Melan-A	0.44	0.12
KIAA2026	KIAA2026	0.61	0.03
RANBP6	RAN-binding protein 6	0.61	0.03
IL33	Interleukin 33	0.33	0.25
TPD52L3	Tumor protein D52-like 3	0.56	0.05
UHRF2	Ubiquitin-like with PHD and ring finger domains 2	0.78	<0.01
GLDC	Glycine dehydrogenase (decarboxylating)	0.28	0.35
GASC1	Lysine (K)-specific demethylase 4C	0.78	<0.01
C9orf123	Chromosome 9 openreading frame 123	0.83	<0.01

Abbreviation: PHD, plant homeodomain.