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RhCG is downregulated in oesophageal squamous cell carcinomas, but expressed in multiple squamous epithelia

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Abstract

To better understand the molecular events underlying the development of oesophageal cancer, we have isolated the genes dysregulated in primary oesophageal cancer tissues using a modified differential display polymerase chain reaction (DD-PCR). In the present study, a gene designated *C15orf6* was identified. The *C15orf6* gene, encompassing 25 kb, is composed of 11 exons with a mRNA of 1948 bp. Database searching showed that *C15orf6* was 100% homologous to the Rh type C-glycoprotein (*RhCG*) with the same open reading frame, but 16 bp longer than *RhCG* at the 5′-end. The gene was highly expressed in human oesophagus, cervix, oral cavity, skin and kidney, but undetectable in the other 14 adult normal tissues examined. Northern blot, RT-PCR and western blot analysis showed that *RhCG*/*C15orf6* was frequently lost or dramatically reduced in primary oesophageal cancer tissues (30/34) compared with the corresponding normal oesophageal mucosa. Three oesophageal-cancer cell lines tested lacked *RhCG*/*C15orf6* expression. Immunohistochemistry revealed that in normal oesophageal tissues, RhCG/C15orf6 was mainly expressed in the plasma membrane of the epithelial cells. In addition, Rh-associated glycoprotein (*RhAG*) expression was also commonly silenced in both oesophageal cancer cell lines (2/3) and primary oesophageal cancer tissues (11/13). To our knowledge, this is the first time that *RhAG* expression has been seen in oesophageal epithelium and extends the functional role of the RhAG protein beyond the erythrocyte. These data suggest that inactivation of *RhCG*/*C15orf6* and *RhAG* occurs frequently during the development of human oesophageal cancer.

Keywords: Oesophageal squamous cell carcinoma; RhCG; Downregulation; Squamous epithelia

1. Introduction

Oesophageal cancer is characterised by a remarkable variation in incidence according to the geographical areas, sex and ethnic group of the patients [1]. The incidence of oesophageal cancer is much higher in some countries or areas, such as North China, France, Iran and South Africa. Several environmental and chemical risk factors, such as dietary factors, nitrosamine exposure and history of injury to the oesophagus, have been found to somehow contribute to the high incidence of this malignancy in Northern China [2,3]. Meanwhile, genetic epidemiology investigations have suggested that susceptibility gene(s) may play a role in the development of oesophageal cancer in this area [4,5]. Our recent study showed that some interactions between genes and environmental risk factors might contribute to the genesis of oesophageal cancer [6]. To further understand the molecular basis underlying oesophageal cancer development, we have used differential display polymerase chain reaction (DD-PCR) to compare the pattern of gene expression between primary oesophageal cancer tissues and the corresponding normal oesophageal mucosa. Multiple genes, known and novel, were found to be dysregulated in all three pairs of primary oesophageal cancer tissues examined [7–10].

Rh (rhesus) blood group antigens are well known as the proteins expressed in the red cell membrane. Recently, Liu and colleagues [11] reported novel non-erythroid Rh

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homologues, human RhCG and mouse Rhcg. Despite sharing a notable similarity to the erythroid forms, including the 12-transmembrane topological fold, RhCG is distinct in chromosome location, genomic organisation, promoter structure and tissue-specific expression. *RhCG* maps to 15q25 of human chromosome 15, and has 11 exons and a CpG-rich promoter. It is mainly expressed in the kidney and testis. *In vitro* translation and *ex vivo* expression studies have shown that RhCG carries a complex *N*-glycan, probably at the ^{48}NLS^{50} sequon of exoloop 1. Data suggests that RhCG is a novel polytopic membrane glycoprotein that may function as an epithelial transporter maintaining normal homeostatic conditions in the kidney and testis.

To date, four Rh members have been reported, including the *RhAG*, *RhCE*, *RhD* and *RhCG* genes. Rh proteins are essential for the integrity and functions of the erythrocyte membrane. Dozens of deletions or mutations of Rh gene loci were identified in Rh_{null} disease, a rare genetic recessive disorder of the erythrocytes [12–19]. Rh blood group antigens, composed of multiple protein complexes including Rh proteins and CD47, LW and glycophorin B via protein interactions, were collectively lost or severely reduced on erythrocytes from Rh_{null} patients, who suffer from a varying degree of chronic haemolytic anaemia and spherostomatocytosis [18,19]. Rh proteins were previously considered to be erythroid-specific and confined to higher vertebrates. However, recent studies have identified RhAG homologues in *Caenorhabditis elegans* and *Geodia cydonium*, suggesting that they may have some unidentified roles that are not confined to erythrocytes [20,21].

In this article, we describe a new oesophageal squamous cell carcinoma-related cDNA, designated *C15orf6*, with 100% identity to RhCG with the same open reading frame but 16 bp longer than RhCG at the 5′-end. We examined RhCG/C15orf6 expression in primary oesophageal cancer tissues and investigated the functional role of RhAG in human epithelium, e.g. oesophageal epithelial cells.

2. Materials and methods

2.1. Cell lines and tissues

Human oesophageal squamous cancer cell lines EC109, EC8712 and EC9706 were established in our laboratory [22,23]. The cell lines were cultured in M199 with 15% (v/v) fetal bovine serum. Fresh tissues, including 34 oesophageal squamous cell carcinomas, matched adjacent normal epithelia 4–5 cm away from malignant tissues and five other normal squamous epithelia (oral cavity mucosa, tongue mucosa, oropharyngeal mucosa, penis skin, cervix and vagina), were procured from surgical resection specimens collected by the Pathology Department in the Cancer Hospital in Anyan, Henan Province and the Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. There were 22 male and 12 female patients with oesophageal cancer. Ages ranged from 38 to 70 years. All the patients received no treatment before surgery. Nineteen carcinomas were clinical stage II, and 15 stage III. Twelve tumours were histopathologically grade I, 14 grade II and eight grade III. Primary tumour regions and the corresponding normal oesophageal mucosa from the same patients were separately excised by experienced pathologists, and immediately placed in liquid nitrogen until use. In addition, three oesophageal squamous carcinomas from Biochain (human oesophagus tumour multisample mRNA northern blot) were also analysed.

2.2. RNA extraction

Total RNA was extracted from the cells and tissues using a Trizol reagent according to the manufacturer's recommendations. Messenger RNA from four normal oesophageal mucosa was isolated using PolyATract protocol (Promega). All total RNAs were digested using RNase-free DNase (Boehringer Mannheim) to remove the residual genomic DNA before use in the cDNA synthesis.

2.3. Differential display PCR

DD-PCR was carried out using cDNAs from three primary oesophageal carcinomas (diagnosed as T2G1, T3G2 and T3G3, respectively) and the corresponding normal oesophageal epithelia as previously described with the following modifications in Ref. [24]. A mixture of $dT_{15}N$ (N = A, G and C) instead of the traditional single $dT_{15}N$ was used in the reverse transcription and DD-PCR reactions. Briefly, first-strand cDNAs were synthesised in a 30-μl reaction containing 5 μg of total RNA, 10 μM $dT_{15}N$ (N = A, G and C) using SuperScript Preamplification System (Gibco). DD-PCR was performed in a 20-μl reaction containing 1 μl of first-strand cDNA, 1×PCR buffer, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates (dNTPs), 1.0 μM arbitrary 10-mer primers (Operon), 3 μM of mixture of $dT_{15}N$ (N = A, G and C) and 1U Taq DNA polymerase (Gibco). PCR conditions were heated at 95 °C for 1 min, followed by two cycles of 95 °C for 15 s, 39 °C for 4 min, and 72 °C for 2 min, and then 32 cycles of 95 °C for 15 s, 39 °C for 2 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The DD-PCR products were resolved on a 6% denatured sequencing gel. The silver staining and recovery of DNA from the stained gel were performed as previously described in Ref. [25].

2.4. Sequence analysis

PCR-amplified cDNA fragments were cloned into the pGEM-T easy vector (Promega). Plasmid DNA was

prepared using the Wizard Miniprep Purification System (Promega). The sequencing reactions were performed by TaKaRa Corp (Dalian City, China). Homology of the cDNA sequences to other nucleic acids and proteins was determined using the National Center for Biotechnology Information BLAST, PSI-BLAST and BLAST2 database search algorithms, respectively. Alignment of the predicted C15orf6 protein with RhAG and RhCG proteins was done using the ClustalW (1.7) program at http://curatools.com/curatools.html.

2.5. 5′-Rapid amplification of cDNA ends (RACE)

In order to characterise the 5′ terminus of the *C15orf6* cDNA, a modified RACE was carried out with a gene-specific primer (GSP) and a designed oligonucletide adaptor. In brief, 1 μg of placental poly A^+ RNA (Clontech) was used for the first-strand cDNA synthesis. A double-stranded cDNA pool was generated using the CapFinder PCR cDNA synthesis protocol (Clontech), purified twice on a Chroma spin TE-400 column (Clontech). The 5′ terminus of the designed oligonucletide adaptor was complementary to the generic adaptor site in the CapFinder system, while the 3′ extension was non-complementary. GSP extension and ligation of the designed oligonucletide adaptor was done in a 50 μl buffer containing 50 ng CapFinder adaptor-ligated double-stranded cDNA, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.8), 4 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 0.5 μM of designed oligonucletide adaptor, 0.5 μM of the *C15orf6* GSP primer, the four dNTPs (each at 200 μM) and 2 U of Vent (exo-) DNA polymerase (New England Biolabs). The reaction mixture was denatured at 94 °C for 40 s and incubated at 68 °C for 7 min, then 5.6 μl of 10× Tsc incubation buffer was added and 5 U of Tsc DNA ligase was added (Boehringer Mannheim) and the incubation continued at 68 °C for 5 min in a 9600 PCR system. RACE products were purified once using a Chroma spin TE-400 column (Clontech). Complementary DNA ends were then amplified by using a gene-specific primer and a primer complementary to the 3′ extension of the designed oligonucletide adaptor.

The putative full-length cDNA of *C15orf6* was deduced according to the 5′-RACE sequence and the sequence homologous to *RhCG*, which was confirmed by an end-to-end PCR using the Advantage-HF cDNA PCR kit (Clonetech).

2.6. Northern blot analysis

Twenty micrograms of total RNAs were resolved on 1.2% agarose-formaldehyde gels and transferred to Zeta-Probe Nylon membranes (Bio-Rad). Adult normal multiple tissue northern blot membranes I and II and human oesophageal tumour multisample mRNA northern blot membrane were purchased from Biochain. The *C15orf6* and *β-actin* cDNAs were radiolabelled with the Prime-a-Gene labelled system (Promega) in the presence of [^{32}P]-deoxycytidine (dCTP) and purified using a G25 spin column. Hybridisation was carried out at 65 °C overnight in 0.25 M sodium phosphate (pH 7.2)–7% (w/v) sodium dodecyl sulphate (SDS). Membranes were washed twice at 65 °C in 20 mM sodium phosphate (pH 7.2)–5% SDS and twice in 20 mM sodium phosphate (pH 7.2)–1% (w/v) SDS. The washed membrane was radiophotographed at −70 °C for 15–50 h. For multiple tissue blot membranes and membrane blottings of matched oesophageal cancer tissues, ethidium bromide staining bands of 28S and 18S rRNA were used to confirm comparative RNA loading.

2.7. RT-PCR analysis

RT-PCR was done in a 30-μl reaction containing 1 μl of first-strand cDNA, 1×PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM primers of *C15orf6* and *α-tubulin*, and 1.5 U Taq DNA polymerase (Gibco). PCR was performed by heating at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min. RT-PCR for *RhAG* was done as for *C15orf6* except using 0.5 μM primers for RhAG, 0.2 μM *β-actin* as internal control, and an increasing cycle number of 33. The RT-PCR products were resolved by 2% (w/v) agarose gel electrophoresis. The length of the PCR products of *C15orf6*, *α-tubulin*, *RhAG* and *β-actin* were 106, 410, 493 and 209 bp, respectively. The sequences of primers used were as follows: *C15orf6*: 5′-GTGAACCTGAGCT CTCCCAG-3′ and 5′-ACCCAGGGAGCATAGGA-GAT-3′; *α-tubulin*: 5′-CTCATCACAGGCAAGGAAG GAT-3′ and 5′-TTAAGGTTAGTGTAGGTTGGGC-3′; *RhAG*: 5′-TCCAGCTTCCTTTCCCATTA-3′ and 5′-CGTTGCAAATAGTCAACATGC-3′; *β-actin*: 5′-CG TGGACATCCGTAAAGACC-3′ and 5′-ACATCTGC TGGAAGGTGGAC-3′.

2.8. Preparation of antibody

Polyclonal anti-RhCG/C15orf6 antibody was generated by immunising rabbits with 18 amino acid (aa) polypeptide (Ac-RRNLEQSKERQNSVYQSC-amide) in complete and incomplete Freund's adjuvant.

2.9. Immunohistochemistry

Serial sections (5 μm thick) of formalian-fixed and paraffin-embedded biopsy samples were cut, and one was stained by haematoxylin-eosin to confirm the histopathological diagnosis. Consecutive sections were processed for the immunohistochemical analysis. Sections were immersed in xylene to remove the paraffin

and then rehydrated through graded alcohol. Epitope retrieval was performed by heating at 95 °C for 30 min in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubating the sections in 3% (v/v) hydrogen peroxide for 5 min. After blocking of non-specific binding by pre-incubation with 1.5% (v/v) normal goat serum for 30 min, primary antibody anti-RhCG/C15orf6 rabbit serum (1:500) was added for 2 h at 37 °C. For detection, horseradish peroxidase-labelled anti-rabbit goat serum was applied as the secondary antibody for 30 min at 37 °C, followed by incubation with DAB solution. After washing with water, the sections were then counterstained with haematoxylin.

2.10. Western blot analysis

Cancer tissues and matched normal mucosa of the oesophagus were lysed in freshly prepared extraction buffer (10 mM Tris–HCl, pH 7.0, 140 mM sodium chloride, 3 mM magnesium chloride, 0.5% (v/v) Nonidet (N)P-40, 2 mM phenylmethylsulphonyl fluoride (PMSF), 1% (w/v) aprotinin and 5 mM dithiothreitol) for 20 min on ice. Proteins (100 μg/lane), resolved on a 10% (w/v) SDS–polyacrylamide gel, were transferred onto NC membrane, and analysed using polyclonal rabbit anti-RhCG/C15orf6 antisera.

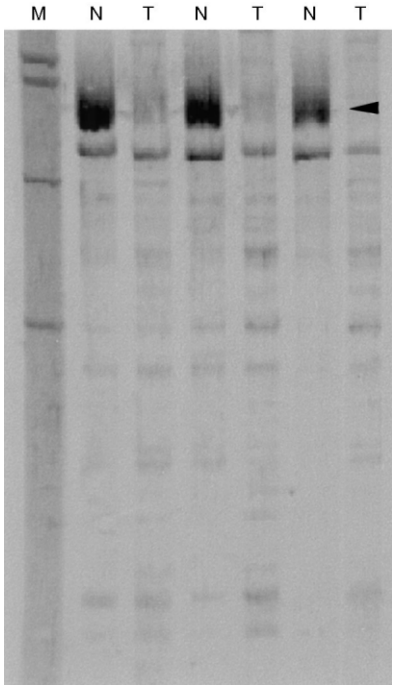

Fig. 1. mRNA differential display of three oesophageal cancer tissues (T) and matched adjacent oesophageal mucosa (N). DD-PCR was done with the arbitrary 10-mer primer OPA5 (5′-AGGGGTCTTG-3′) and a mixture of $dT_{15}N$ (N = A, G and C). PCR products were resolved on 6% denatured sequencing gel and detected by silver-staining. Arrows indicates the recovered band designated *C15orf6*. M: ϕX174 DNA/HaeIII marker.

3. Results

3.1. Identification of C15orf6 cDNA

To identify genes dysregulated in oesophageal cancer, three primary oesophageal cancer tissues were compared with the corresponding normal oesophageal epithelia. The pattern of gene expression was extremely similar for either all three primary cancer tissues or adjacent normal oesophageal epithelium, indicating a low variation of gene expression between different individuals and the high reproducibility of the modified DD-PCR. With the OPA5 arbitrary 10-mer primer (5′-AGGGGTCTTG-3′) in combination with a mixture of $dT_{15}A$, $dT_{15}G$ and $dT_{15}C$, one cDNA band of approximately 250 bp was found to be not expressed or barely expressed in all three oesophageal cancer tissues, while it was highly expressed in matched adjacent oesophageal mucosa (Fig. 1). Sequence analysis showed that the cDNA was 100% identical to the Rh type C glycoprotein (*RhCG*, NM_016321), which has been reported to be expressed in the testis and kidney [11]. By combining the RT-PCR and RACE methods, we identified the 1948-bp full-length cDNA, designated *C15orf6*. Since we cannot get normal adult oesophageal epithelium, we instead used the normal oesophageal mucosa adjacent to the cancer portions for preparing mRNA. To exclude the possibility that the *C15orf6* gene might be mutated in the cancer-adjacent normal oesophageal mucosa, the foetal oesophageal cDNA was then used as template for amplification of 1.9-kb *C15orf6* cDNA corresponding to nucleotide positions 2–1930. Two independent cDNA clones were selected for sequencing. No sequence difference between two clones was found. *C15orf6* was 100% homologous to the Rh type C-glycoprotein (*RhCG*, NM_016321), but 16 bp longer than *RhCG* at the 5′-end. As shown in Fig. 2, the 1948-bp cDNA sequence includes 40 bp of 5′ and 469 bp of 3′ untranslated sequences and 1440 bp of open reading frame (including the stop codon) encoding a 479-aa protein (GenBank accession number AF081497). Blast searching indicated that the full-length cDNA of *C15orf6* was 100% identical to part of BAC clone RP11-429B14 located on human chromosome 15 (GenBank accession number: AC013391). This indicated that our sequence result of *C15orf6* was correct, and the additional 16 bp at 5′-terminus was specific for *C15orf6*. Alignment analysis showed that the genomic DNA of *C15orf6* encompassed 25 172 bp in length (GenBank accession numbers: AF284446, AF185277), with 11 exons and 10 introns. The deduced C15orf6 protein is the same as that of the *RhCG* gene, which is encoded by the exons 1–10, and the last exon does not encode for any amino acids.

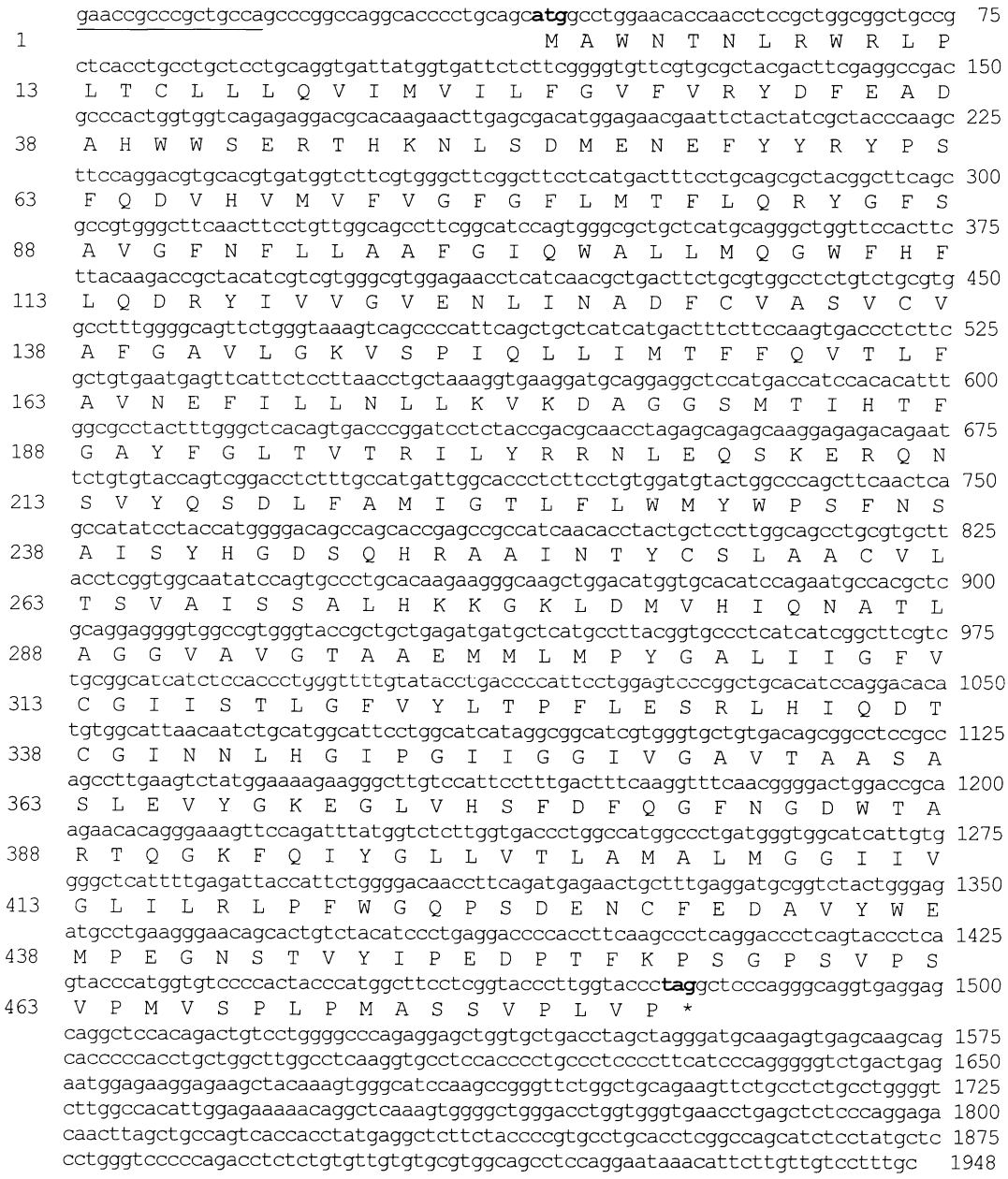

Fig. 2. The putative full-length cDNA and the predicted amino acid sequence of the *C15orf6* gene. The sequence has been deposited in the GenBank (accession number AF081497). The complete cDNA of *C15orf6* is 16bp (underlined) longer than RhCG in the 5′ untranslated region.

3.2. *Distribution of* RhCG/C15orf6 *expression in human adult normal tissues*

Northern blot analysis was used to determine the pattern of *RhCG/C15orf6* expression in human adult normal tissues. As shown in Fig. 3, one *RhCG/C15orf6* transcript of 2.0 kb, corresponding well to the isolated cDNA sequence of 1948 bp, was detected only in human oesophagus and kidney, but not in the other 14 adult tissues examined, including stomach, small intestine, colon, uterus, placenta, bladder, adipose, heart, brain, liver, lung, pancreas, spleen and skeletal muscle.

3.3. *Immunohistochemistry of RhCG/C15orf6*

In normal oesophageal mucosa, more than two thirds of the squamous epithelium predominately expressed RhCG/C15orf6, and the basal cells and the superficial cells exhibited very weak staining (Fig. 4a). The immunohistochemical reaction was mainly confined to the plasma membrane of the normal epithelial cells. Of the cancer tissues examined, well and moderately differentiated carcinomas presented discontinuous and weaker expression of RhCG/C15orf6 (Fig. 4b). In poorly differentiated carcinomas, immunohistochemical staining was very faint or completely negative.

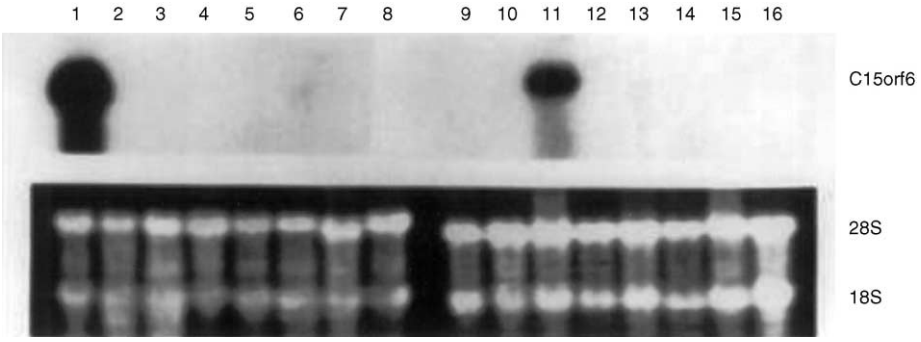

Fig. 3. Distribution of *C15orf6* mRNA in normal human adult tissues. *C15orf6* cDNA was labelled with ^{32}P-dCTP and hybridised to two multiple adult normal tissue northern blots (Biochain). The ethidium bromide staining bands of 28S and 18S rRNAs were shown to confirm comparative loading of the total RNAs. (1): oesophagus; (2) stomach; (3) intestine; (4) colon, (5) uterus; (6) placenta; (7) bladder; (8) adipose; (9) heart; (10) brain; (11) kidney; (12) liver; (13) lung; (14) pancreas; (15) spleen (16) skeletal muscle.

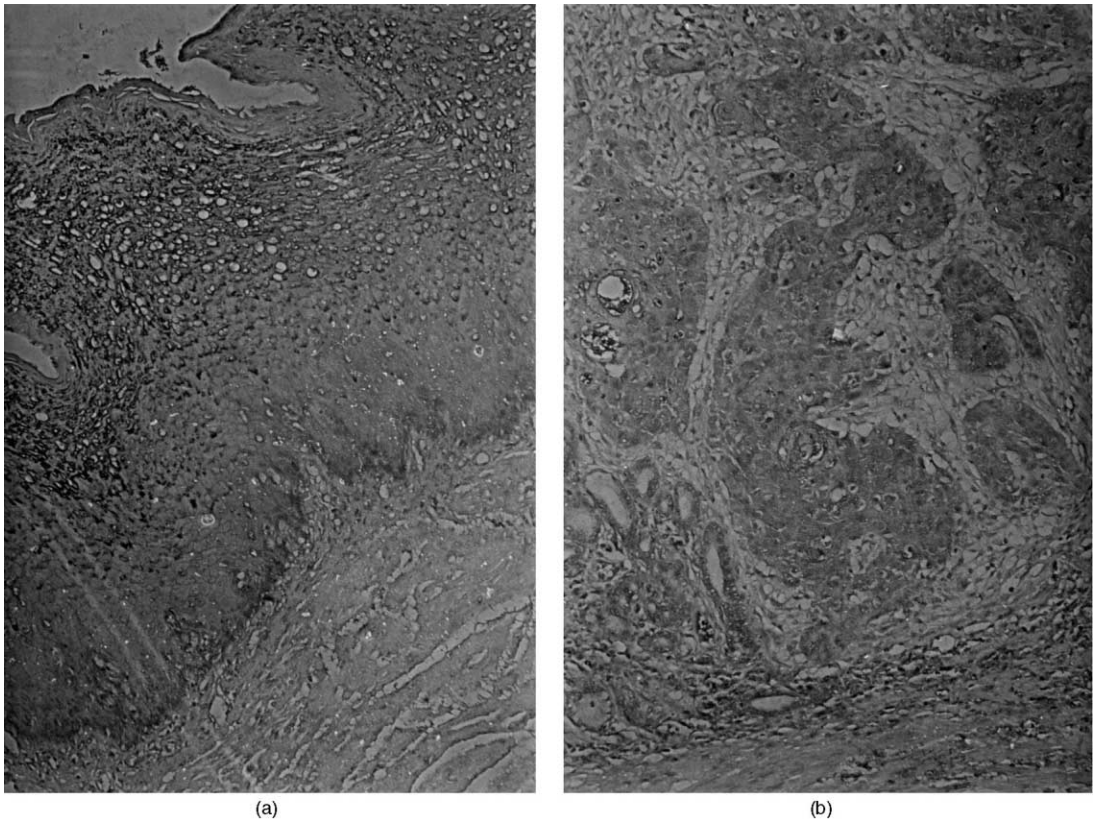

Fig. 4. Immunohistochemistry staining showed that more than two-thirds of the normal squamous epithelium of the oesophagus predominately expressed RhCG/C15orf6, and the basal cells and the superficial cells exhibited very weak staining (a). Compared with the adjacent epithelia, discontinuous and weaker expression of RhCG/C15orf6 was observed in more differentiated carcinomas (b).

We also investigated five other types of squamous epithelia tissues, including oral cavity mucosa, tongue mucosa, oropharyngeal mucosa, penis skin, cervix and vagina. The results showed that RhCG/C15orf6 were expressed in all of these epithelial tissues, with the strongest staining in the basal layer of the vaginal epithelium (Fig. 5a–e).

3.4. *Expression of* RhCG/C15orf6 *and* RhAG *is frequently lost in oesophageal cancer*

Expression of *RhCG/C15orf6* in four normal and cancer tissues was analysed by northern blotting. *RhCG/C15orf6* was not detectable or dramatically

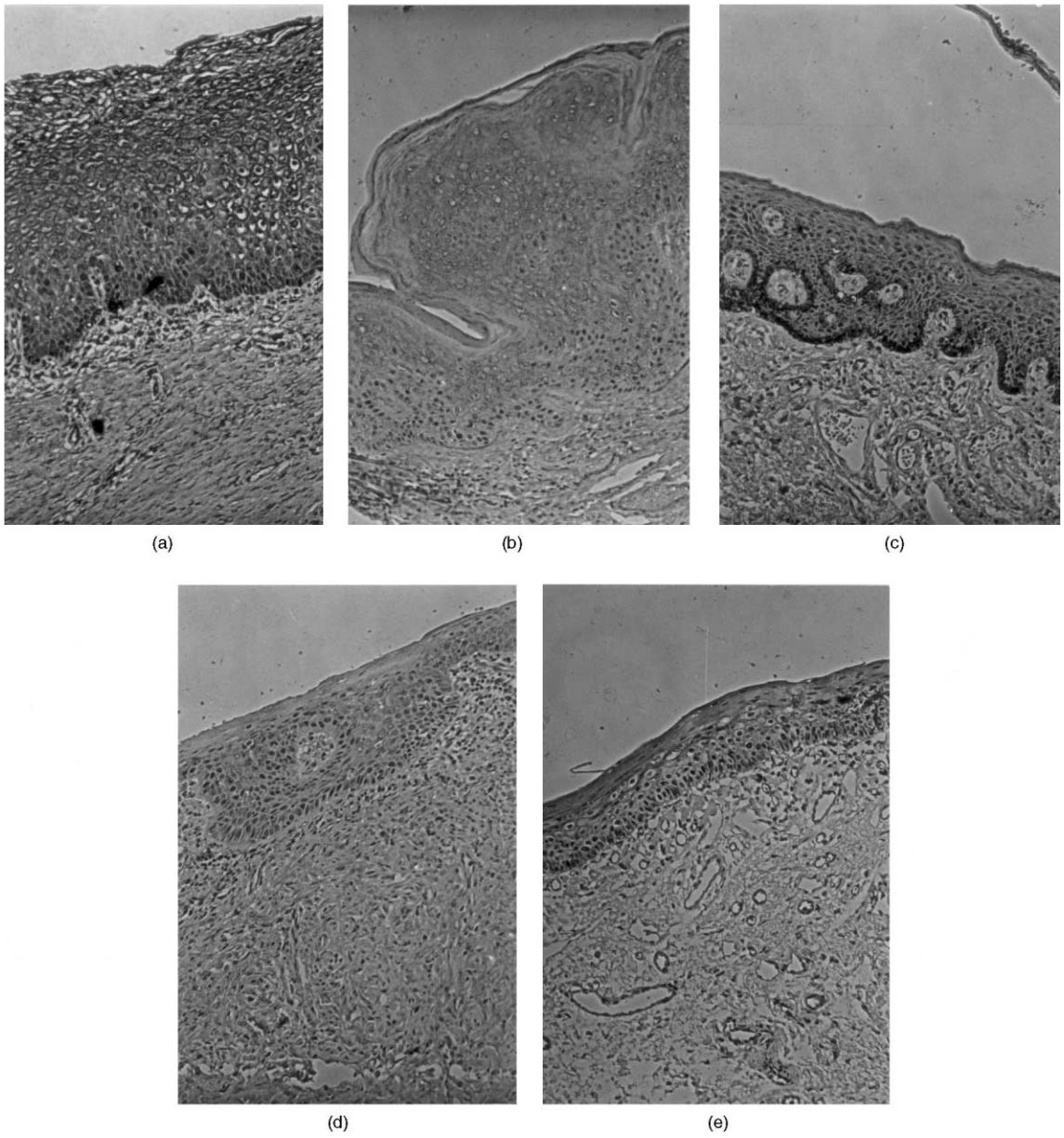

Fig. 5. Immunohistochemistry staining showed that RhCG/C15orf6 is expressed in multiple normal squamous epithelia. (a) cervical epithelia; (b) penis skin; (c) vaginal epithelia; (d) tongue epithelia; (e) oropharyngeal epithelia. Note that tongue epithelia presented a similar pattern of RhCG/C15orf6 expression to the oesophagus, i.e. stronger in more differentiated cells and weaker in the basal cells and the superficial cells. In addition, unlike the five other squamous epithelia, vaginal epithelia (c) displayed prominent immunohistochemistry staining in the basal cells.

reduced in all oesophageal cancer tissues, while a transcript of approximately 2.0 kb was found to be expressed at a high level in all if the matched adjacent normal oesophageal mucosa (Fig. 6a). Analysis of the NBA blot from Biochain demonstrated that expression of *RhCG*/*C15orf6* was predominant in the normal oesophageal epithelium, but very faint in the three oesophageal epithelium carcinomas. *RhCG*/*C15orf6* was not expressed in the normal lung tissue (Fig. 6b). Then, 26 pairs of matched human oesophageal cancer tissues/adjacent epithelial tissues and three oesophageal squamous cell lines were examined for *RhCG*/*C15orf6* expression by semi-quantitative duplex RT-PCR, in which *α-tubulin* was employed as an internal control for both cDNA quality and efficiency of the PCR amplification. RT-PCR analysis indicated that expression of *RhCG*/*C15orf6* was undetectable or barely detectable in 22 of 26 oesophageal cancer tissues, and no expression of *RhCG*/*C15orf6* was found in all three oesophageal cancer cell lines. A representative result of the RT-PCR analysis is shown in Fig. 7. To confirm the expression status, the whole coding sequence of *RhCG*/*C15orf6* was amplified for some of the matched human oesophageal cancer tissues/normal mucosas. Consistent results were acquired using two primer sets

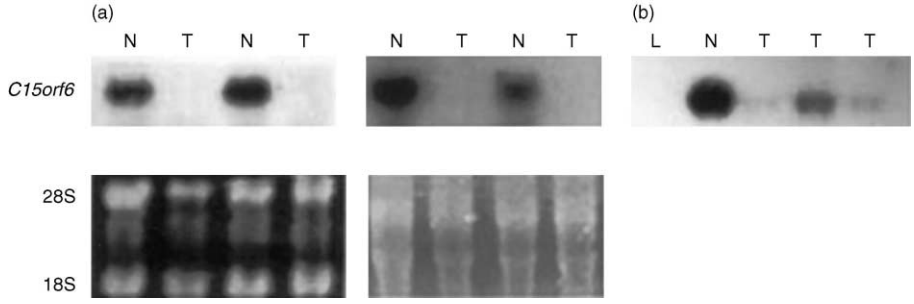

Fig. 6. Northern blot analysis of *C15orf6* transcript in matched oesophageal cancer tissues (T) and adjacent histologically normal tissues (N). Total RNAs (20 μg/lane) were isolated, electrophoresed, blotted and hybridised with ^{32}P-dCTP labelled *C15orf6* probe as described in Section 2. Bands of 28S and 18S rRNAs were used to normalise the human *C15orf6* hybridisation signals (a). NBA blot from Biochain was hybridised by the same probe (b). L, normal lung tissue; N, normal oesophageal epithelium; T, oesophageal carcinoma.

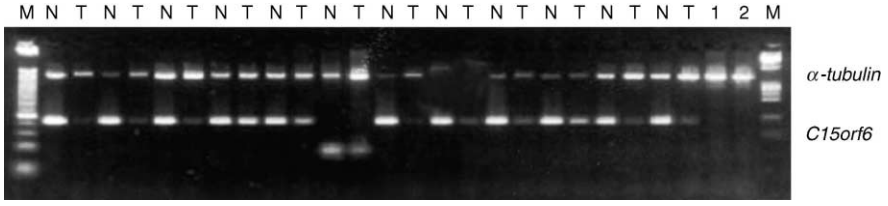

Fig. 7. Representative RT-PCR analysis results of *C15orf6* in matched oesophageal cancer tissues and cell lines. Duplex PCR was performed by mixing 0.5 μM *C15orf6* primers and *α-tubulin* primers as internal control. The RT-PCR products were resolved on 2% agarose gel. T and N indicate samples of tumour tissues and adjacent histologically normal tissues, respectively. 1 and 2: oesophageal cancer cell lines EC9706 and EC8712; M: 100 bp DNA ladder.

to amplify the 3′ untranslated region and whole coding sequence of *RhCG*/*C15orf6*.

Western blot analysis further confirmed that RhCG/C15orf6 was downregulated in oesophageal cancer tissues, whereas it was highly expressed in the matched adjacent epithelia (Fig. 8).

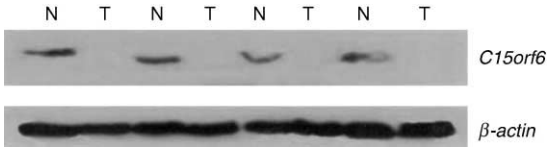

Fig. 8. Western blotting confirmed the downregulation of C15orf6 protein in oesophageal cancer (T) compared with the corresponding adjacent epithelia (N).

Overall, the expression of RhCG/C15orf6 was greatly reduced or completely absent in 30 out of 34 primary cancer tissues compared with the corresponding adjacent normal oesophageal mucosa, suggesting that the lack of RhCG/C15orf6 expression was common in primary oesophageal cancer tissues.

The high sequence homology between *RhCG*/*C15orf6* and *RhAG* prompted us to determine if the expression of *RhAG* was also defective in human oesophageal cancer tissues, as well as cancer cell lines. RT-PCR analysis showed that *RhAG* was expressed in adjacent normal epithelia, but frequently lost (11/13) in cancer tissues. Besides, *RhAG* was undetectable in two of three oesophageal cancer cell lines, but expressed in the oesophageal cancer cell line EC9706 (Fig. 9).

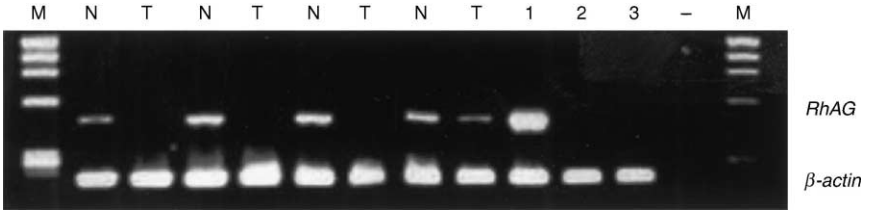

Fig. 9. Representative RT-PCR analysis results of *RhAG* in matched oesophageal cancer tissues and cell lines. Duplex PCR was performed by mixing 0.5 μM RhAG primers and 0.2 μM *β-actin* primers as an internal control. T and N indicate samples of tumour tissues and adjacent histologically normal tissues, respectively. 1–3: oesophageal cancer cell lines EC9706, EC8712 and EC109; –: negative control using water as the PCR template; M: ϕX174 DNA/HaeIII marker.

4. Discussion

RhCG is a newly found gene encoding a plasma-membrane protein sharing strong similarity to Rh proteins in amino acid sequence and 12-transmembrane topology. It has been suggested RhCG may act as an epithelial transporter maintaining normal homeostatic conditions in the kidney and testis [11]. The results of multiple tissue northern blot and immunohistochemistry with many kinds of squamous epithelia revealed that *RhCG*/*C15orf6* expression was restricted in certain tissues, such as the kidney, oesophagus and other kinds of squamous epithlia examined. Expression of *RhCG*/*C15orf6* in the kidney confirmed the previous reports by Liu and colleagues. The existence of the RhCG/C15orf6 protein in all the squamous epithelia examined suggested that the expression of this gene may be a common event in normal squamous epithelial cells.

RT-PCR analysis showed that *RhAG*, like *RhCG*/*C15orf6*, was also expressed in oesophageal tissues. So far, *RhCE* and *RhD* genes have been found to be expressed in erythroid cells only. The expression of the *RhAG* gene in squamous epithelium was identified here for the first time, although at a lower level than *RhCG*/*C15orf6* gene. Our results provide further evidence that the RhAG and RhCG/C15orf6 proteins play an important role beyond that in erythrocytes, e.g. in oesophageal epithelium as identified in this study.

In the present study, the oesophageal cancer tissues examined were obtained from two geographical regions of China; the high-incidence area of Anyang and low-incidence area of Beijing. Several environmental and chemical risk factors, such as dietary factors, nitrosamine exposure and history of injury to the oesophagus, have been found to somehow contribute to the high incidence of this malignancy in North China [26,27]. Since loss or reduction of *RhCG*/*C15orf6* expression was identified in most cancer tissues from either the high or low-incidence areas, we speculate that downregulation of the *RhCG*/*C15orf6* gene is not a downstream event affected by the environmental and chemical risk factors found in the high-incidence area. Loss or reduction of *RhCG*/*C15orf6* expression was common in oesophageal squamous carcinomas, both stages II and III. However, as we did not have tumour tissues at clinically earlier stages, it remains to be identified whether down-regulation of *RhCG*/*C15orf6* is an early event in the malignant transformation of the oesophagus.

Frequent loss or reduction of expression of *RhCG*/*C15orf6* and *RhAG* infers an important role for inactivation of these two genes in oesophageal cancer. The functional significance of the inactivation of Rh proteins in the human malignancies has also been derived from case observations. Firstly, loss of Rh antigens in the erythrocyte membrane was found to be related to chromosome aberrations in some patients with myeloid leukaemia [28]. Secondly, a somatic mutation in the RHD gene was identified in a patient with chronic myelocytic leukemia (CML) whose Rh phenotype was changed from Rh-positive to Rh-negative during the three-years of study [12]. In Rh_{null} disease, point mutations of the *RhAG* gene, mainly in the conserved transmembrane domains, have been found to account for the loss of the RhAG protein and thus loss of Rh proteins on the erythrocyte membrane in Rh_{null} patients. However, we have not yet identified any mutations of the *RhCG*/*C15orf6* and *RhAG* genes in oesophageal cancer cells. PCR analysis indicated that the 3′ untranslated sequences of the *RhCG*/*C15orf6* and *RhAG* genes were both intact in oesophageal cancer cell lines. We also analysed the genomic DNA of matched oesophageal carcinomas and adjacent normal mucosa by Southern blot hybridisation, and no detectable deletion of *RhCG*/*C15orf6* was observed in the tumour tissues (data not shown). Effort should now be made to investigate whether methylation of the regulatory sequence led to the downexpression of *RhCG*/*C15orf6* in oesophageal squamous cell carcinomas.

Little is known about the physiological functions of the *RhCG*/*C15orf6* and *RhAG* genes. Westhoff and colleagues recently reported that RhAG may be an ammonium transporter [29]. Matassi and colleagues showed that the length of both the whole protein and C-terminal of RhAG proteins in different species gradually reduced during the evolution from nematodes to humans [30]. While the length of both the whole protein and C-terminus of the RhCG/C15orf6 predicted protein was 72-aa longer than that of human RhAG protein, but similar to two RhAG proteins (GenBank T18673 and AF183390) in *C. elegans*. Moreover, some expressed sequence tags (ESTs) isolated from mouse kidney (GenBank AI956391, AI875358, AW045171, AW475348, AW012152, AI037123, etc.) shared 84–85% homology to the 5′ sequence of the *RhCG*/*C15orf6* mRNA. These data suggest that *RhCG*/*C15orf6* may be a relatively conserved gene, mainly expressed in squamous epithelia, kidney and testis. In normal squamous epithelia of the oesophagus, oral cavity mucosa, tongue mucosa, oropharyngeal mucosa, penis skin and cervix, RhCG/C15orf6 was more strongly expressed in the more differentiated cells, suggesting that RhCG/C15orf6 expression could be related to the differentiation of these squamous epithelia. Rh_{null} patients with loss or severe reduction of Rh blood group antigens on erythrocytes suffered from a varying degree of chronic haemolytic anaemia and spherostomatocytosis, which indicated that Rh proteins are essential for the integrity and functions of the erythrocyte membrane [18,19]. Based on the high homology of *RhCG*/*C15orf6* to RhAG, we can postulate that RhCG/C15orf6 plays a role in maintaining the structure and functions of the plasma membrane of squamous epithelial cells. Interestingly, RhCG/C15orf6 was predominately expressed in the basal cells of the vaginal

epithelia, which was a different expression pattern compared with the five other squamous epithelia. It is still unclear whether RhCG plays a particular/different role in vaginal epithelium compared with the other squamous epithelia. Future studies are needed to dissect the precise physiological functions of the *RhCG*/*C15orf6* and *RhAG* genes in diverse human squamous epithelia and the relationship between inactivation of the genes and the development of human oesophageal cancer.

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