

# Orexin 2 receptor as a potential target for immunotoxin and antibody-drug conjugate cancer therapy

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**Abstract.** Targeting tumor-specific receptors is a promising approach for cytotoxic agents. The orexin 2 receptor (OX2R) has reportedly been expressed in a few types of cancer, but not in normal cells. This study aimed to explore and assess the expression levels of OX2R in a wide range of cancer cell lines and clinical samples to identify its localization. To analyze OX2R expression, we developed a polyclonal antibody specific to OX2R by immunizing two rabbits with a peptide cocktail. A total of 36 cancer cell lines were employed for reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis, and 221 samples from various tissue arrays were used for the immunohistochemistry of OX2R expression. OX2R was identified in three cancerous cell lines, from the gallbladder, squamous cell carcinoma of the head and neck (SCCHN) and glioblastoma. With clinical samples of tissue arrays, 69/221 (31.2%) samples reacted positively with the OX2R antibody. We confirmed its presence on the cell membrane. In conclusion, OX2R was identified on several cancer cells as well as clinical samples. Further studies with larger numbers of clinical samples are required to confirm the statistical significance of the presence and relationships of OX2R with tumor histology. Results of the current study suggested that OX2R is a potent target for immunotoxin or antibody-drug conjugate (ADC) cancer therapy on OX2R-positive cancer cells.

## Introduction

Cytotoxic drugs are still widely used to treat malignant tumors via tumor cell death by apoptosis (1). One of the most representative cytotoxins is cisplatin, which reacts with target genomic DNA to form DNA adducts (2). Cytotoxins are typically non-selective and this lack of selectivity occasionally results in significant toxicity to normal cells. Moreover, treatment of cancer patients with chemotherapeutic agents frequently allows tumors to acquire a multidrug-resistant (MDR) phenotype (3,4). Overexpression of MDR1, other ATP-dependent transporters, amplification of drug-inactivating enzymes, mutations or modifications of drug targets, alterations in DNA repair machinery and increased resistance to apoptosis cause this resistance (3-5). Toxicities of chemotherapy, along with drug resistance, are major therapeutic limitations that result in poor clinical outcomes in cancer patients.

Targeting of low extracellular pH, elevated enzymes in tumor tissues, the hypoxic environment inside the tumor and tumor-specific antigens expressed on tumor cell surfaces were previously investigated as possible strategies for improved therapeutic outcomes (6-9). Singh *et al* highlighted recent trends in pro-drug and conjugate rationale and a design for cancer treatment, by analyzing comparative accounts of the advantages and disadvantages associated with each approach (10). A variety of receptors related to cellular growth factors or cytokines on tumor cells have been shown to be overexpressed (11), and we believe that targeting these receptors is a promising strategy. For example, the transfection of the tumor necrosis factor (TNF) receptor gene in cancer cells, or the exposure of cancer cells to certain reagents, may increase the expression of TNF receptors, resulting in the enhancement of the cytotoxic effect of TNF (12). Identifying new receptors on tumor cells, in addition to further investigation of therapeutic strategies, is still crucial for cancer treatment.

In the process of finding potential candidate receptors in cancer cells, the orexin 2 receptor (OX2R, also known as hypocretin receptor 2) was found to be a noteworthy target. The orexin family comprises orexins-A and -B, and their corresponding receptors are OX1R and OX2R, respectively. These

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two receptors belong to the seven-transmembraned G-coupled receptor superfamily (13). It has become clear that orexin receptors regulate narcolepsy (14,15). Immunohistochemistry (IHC) analyses have demonstrated that certain peptides that bind to orexin receptors were selectively expressed in the hypothalamus, particularly in the lateral and medial hypothalamic regions (16).

At present, the presence of orexin receptors reported in human cancer cells is limited. The expression of OX1R has been found in cell lines from human colon cancer (17). However, the study pertaining to the expression of OX2R is limited to clinical samples of cortisol-secreting adrenocortical adenomas (18). Moreover, the role of orexin receptors in cancer cells is as yet unknown. Thus, identifying the location of orexin receptors remains a challenge.

OX2R was selected as a possible candidate since the expression levels of OX2R in normal cells are limited (16) and, thus, OX2R may be a cancer cell-specific target. Although a high expression of OX2R has been identified in hypothalamic samples, systemic administration of antitumor drugs targeting OX2R may not interact with the hypothalamus due to the presence of the blood-brain-barrier. In addition, orexin receptors are a well known target in the field of neuroscience, since they closely correlate with narcolepsy and other diseases (14,15). Investigating other functions of OX2R would be helpful in understanding the roles of orexins.

In the present study, following the screening of OX2R expression in a variety of cancer cell lines, we investigated cancer tissue array samples to further examine OX2R expression. In addition, we examined the possibility of OX2R as a target for immunotoxin or antibody-drug conjugate (ADC) cancer therapy.

## Materials and methods

**Materials.** Hepatocellular carcinoma tissue array, ARY-HH0075 was purchased from Folio Biosciences (Columbus, OH, USA). Digestive system disease tissue array, DID381 was purchased from US Biomax (Rockville, MD, USA). Human cancer tissue array, VA2 was purchased from SuperBioChips (Seoul, Korea). All other reagents were of reagent grade quality. All tissue samples were collected under the ethical standards with the donor's complete informed consent under the control of manufacturers.

**Polyclonal antibody against OX2R.** Two New Zealand white rabbits were immunized with three specific peptides of OX2R to obtain a polyclonal antibody for OX2R. Three peptides (CRNWSSASELNETQE, FAHTEDRET VYAWF and C-AVA AEIKQIRARRK) were selected; GenBank reference: CAI19665.1. The immunization period was 64 days and immunization was performed four times during this period. Rabbit serum (10 ml) were purified with a peptide affinity column. The antibody titer of ELISA for CRNWSSASELNETQE was estimated to be 1 in 4,000 and for C-AVA AEIKQIRARRK to be 1 in 16,000. All procedures were performed by Japan Bio Services (Saitama, Japan).

**Reverse transcription polymerase chain reaction (RT-PCR).** Total RNA was extracted according to the manufacturer's instructions (TRIzol, Invitrogen, Carlsbad, CA, USA). Following

quantification of the extracted RNA, first strand complementary DNA (cDNA) of each cancer cell line was synthesized.

OX2R expression was measured by RT-PCR, using 1  $\mu$ g RNA and oligo (dT) as reverse transcription primers. A control reaction, which omitted reverse transcriptase was included to check for the presence of genomic DNA. OX2R was amplified using a Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in a 20- $\mu$ g reaction medium containing 0.1  $\mu$ g of AmpliTaq Gold<sup>®</sup> DNA Polymerase (Applied Biosystems), using the following cycling conditions: 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 60 sec, followed by a 10 min extension at 72°C. The sequences for the sense and anti-sense primers of OX2R and  $\beta$ -actin used were: 5'-caccgtgttcccaggcttag-3', 5'-ttctggctcggatctgctt-3', 5'-cactgtgttgccgtacaggt-3' and 5'-tcataccattggcaatgag-3'. Amplicons were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide and viewed under UV illumination. Gene sequence of OX2R was performed by Invitrogen Japan (Tokyo, Japan).

**Western blotting.** Proteins (30  $\mu$ g) were evaluated by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA, USA), loaded onto a NuPAGE 10% Bis-Tris gel (Invitrogen) and blotted onto a Polyscreen polyvinylidene fluoride (PVDF) transfer membrane (ParkinElmer, Waltham, MA, USA) at 100 V for 1 h in a transfer buffer. The PVDF membranes were incubated with primary monoclonal antibody at a 1:2,000 dilution and with  $\alpha$ -tubulin antibody (ab24246, Abcam, Cambridge, MA, USA) at a 1:20,000 in phosphate-buffered saline (PBS)-0.1% Tween (PBS-T) and 1% bovine serum albumin (BSA) overnight at 4°C. The membranes were washed, incubated with a secondary anti-rabbit horseradish peroxidase-conjugated antibody (GE Healthcare, Fairfield, CT, USA) at a 1:20,000 dilution for 90 min at a room temperature and washed for 60 min with PBS-T. Antibody complexes were visualized using enhanced chemiluminescence (ECL) plus western blotting reagent (GE Healthcare). The images were scanned by LAS-3000 UVmini (Fuji Film, Tokyo, Japan).

**IHC.** IHC was performed using tissue arrays. Tissue arrays were deparaffinized by xylene treatment and washed with an alcohol gradient (from 99 to 50%) and PBS. Arrays were incubated with polyclonal antibody against OX2R at a 1:1,000 dilution overnight at 4°C. Slides were developed with alkaline phosphatase (AP)-goat anti-rabbit IgG (Invitrogen) and stained by BM purple AP substrate (Roche Diagnostics, Rotkreuz, Switzerland). Slides were also lightly counterstained with hematoxylin and eosin. Negative controls in this study were prepared by omitting the primary antibody step.

**Immunohistochemical scoring.** Samples were scored independently by an experienced histopathologist. Immunostaining was scored as ++/+++ (strong), + (moderate) or - (negative).

## Results

**OX2R mRNA expression in cancer cells assessed by RT-PCR.** One of the main objectives of this study was to confirm the expression levels of OX2R in cancer cells. We investigated 36 cancer cell lines originating from 10 different organs, ranging

Table I. Cell lines used for RT-PCR and western blotting screening.

Organ	Cell line
SCCHN	YCUT231, YCUT891, YCUM 862, YCUM911, YCUMs861, YCUL891, Wmm-scc
Glioblastoma multiforme	SN19, U251tg, A172, U373, SF295
Breast	BT-20, MDA-MB-231, ZR-75-1, T47D
Liver	Hep3B, HepG2, PLC/PFR-5, HuH-7
Gallbladder	TGBC1, TGBC2, TGBC14, TGBC44, NOZ, OCUg
Cholangiocarcinoma	MzChA1, MzChA2, OZ, HuCCT-1
Pancreas	PANC1, SU.86.86
Uterine cervix	Hela, HT-29
Oesophageal carcinoma	OE19
Colon	DLD-1

RT-PCR, reverse transcription polymerase chain reaction; SCCHN, squamous cell carcinoma of the head and neck.

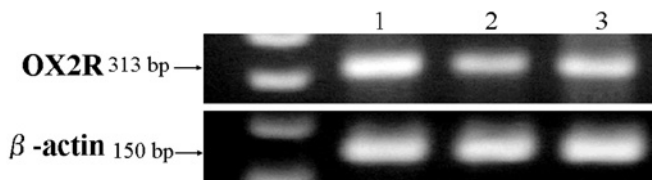


Figure 1. RT-PCR analysis of OX2R mRNA on cancer cells. Total RNA isolated from cancer cell lines (lane 1, TGBC2; lane 2, SF295 and lane 3, YCUM862) was reverse transcribed to cDNA and then assessed by PCR using specific primers as described in Materials and methods.  $\beta$ -actin was used as an internal control. Arrows show the location of amplified PCR products of OX2R and  $\beta$ -actin. OX2R, orexin 2 receptor; RT-PCR, reverse transcription polymerase chain reaction; cDNA, complementary DNA.

from glioblastoma to uterine cervix, and screened all 36 cell lines to assess this objective (Table I). Total RNA isolated from cancer cell lines was transcribed to cDNA, and then assessed by PCR as described in Materials and methods. Primer sequences and RT-PCR conditions were optimized by referring to other investigations analyzing the expression of OX2R (19,20) and, through several trials, to obtain a clear single band.

Of the 36 cell lines, three cell lines, originating from the gallbladder (TGBC2), glioblastoma (SF295) and squamous cell carcinoma of the head and neck (YCUM862), expressed OX2R mRNA (Fig. 1). In addition, we performed gene sequences of PCR products to confirm its homology to OX2R. The homology between PCR products and OX2R was 98%. We also screened for the expression of OX1R mRNA, but this was not identified in any of the cell lines tested in this study (data not shown).

**OX2R protein expression in cancer cells assessed by western blotting.** To investigate the expression of OX2R protein on cancer cells, we performed western blot analyses on the same cancer cell lines. Since antibodies against OX2R available for both western blotting and IHC were limited, we produced a polyclonal antibody against OX2R. We immunized two rabbits with three peptides specific to OX2R and purified serum as described in Materials and methods. Fig. 2 shows that all 3 cancer cell lines originated from the gallbladder (TGBC2), glioblastoma (SF295) and squamous

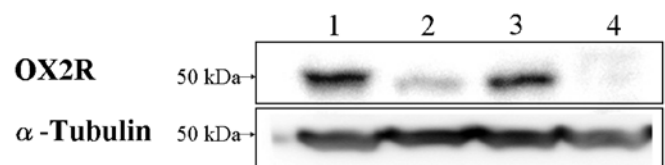


Figure 2. Expression levels of OX2R protein on cancer cells, which were found to be positive for OX2R mRNA upon RT-PCR analysis. Total cell lysates from cancer cells (lane 1, TGBC2; lane 2, SF295 and lane 3, YCUM862) were examined by western blot analysis as described in Materials and methods using specific antibody against OX2R. The extract from the OE19 cell line (lane 4), which was negative for OX2R mRNA expression in RT-PCR analysis was used as a negative control sample.  $\alpha$ -tubulin was used as the loading control. Arrows show the location of the molecular weight for OX2R and the  $\alpha$ -tubulin protein. OX2R, orexin 2 receptor; RT-PCR, reverse transcription polymerase chain reaction.

cell carcinoma of the head and neck (YCUM862), which were positive for OX2R mRNA expressed the protein of OX2R. Conversely, OX2R was not detected in the OE19 cell line, which did not express the mRNA of OX2R in RT-PCR analysis and was used as a negative control (Fig. 2). This result indicates that the three cell lines express OX2R.

**OX2R expression in clinical tissue array samples.** Based on the results of the mRNA and protein expression analysis, we performed an IHC analysis to analyze localization of OX2R on multiple organs and its frequency of expression among clinical cancer using the polyclonal antibody against OX2R shown at the western blot analysis stage. We prepared 221 clinical samples from the three tissue arrays purchased, which enabled us to obtain insights on the objectives described previously. Details of tissue arrays and protocols are described in Materials and methods.

As shown in Table II, 69 of the 221 samples expressed OX2R, including 8 samples with strong staining and 61 samples with moderate staining. The remaining 152 samples did not react with the OX2R antibody. We observed that the expression of OX2R was located on the cell membrane, where receptors should be located, as shown in Fig. 3. These results suggest that 31.2% of clinical cancer samples express OX2R on their

Table II. Summary of OX2R immunohistochemistry analyses in a variety of cancers using three tissue arrays.

Organ	No. of patients	Staining intensity			Positive samples (%)
		-	+	++/+++	
Liver	80	71	7	2	11
Stomach	34	16	17	1	53
Colon	20	13	7	0	35
Salivary gland	15	8	6	1	47
Larynx	15	7	7	1	53
Rectum	9	6	2	1	33
Lung	9	8	1	0	11
Esophagus	6	6	0	0	0
Small intestine	6	0	5	1	100
Nasopharynx	6	4	2	0	33
Pancreas	6	3	3	0	50
Gallbladder	5	2	3	0	60
Tongue	2	2	0	0	0
Maxilla	2	0	1	1	100
Bile duct	2	2	0	0	0
Mandible	1	0	1	0	100
Lower lip	1	1	0	0	0
Tonsil	1	1	0	0	0
Anus	1	1	0	0	0
Total	221	151	62	8	

OX2R, orexin 2 receptor.

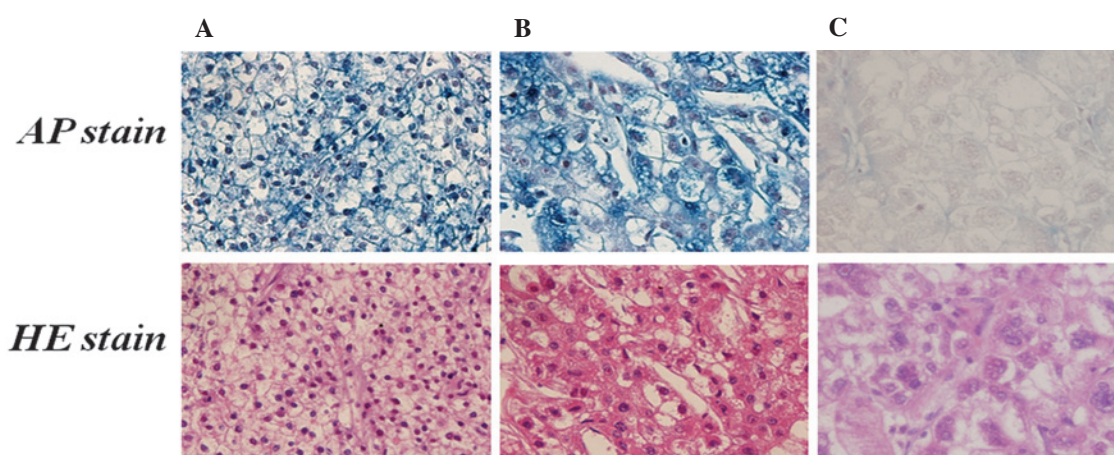


Figure 3. Immunohistochemistry analysis for OX2R expression in various types of cancer. Tissue specimens on arrays were stained with hematoxylin and eosin (H&E) or with alkaline phosphatase (AP), which binds specific polyclonal antibodies to OX2R. (A) Images from sample Folio ARY-HH0075 C6 (hepatocellular carcinoma; original magnification, x200). (B) The magnified immunohistochemical image shows the staining for OX2R is significantly limited on the cell membrane. (Folio ARY-HH0075 G3 sample; original magnification, x400). (C) Images of Folio ARY-HH0075 C4 as a negative control (original magnification, x400). OX2R, orexin 2 receptor.

cell membrane. It is also assumed that cancer organs such as the stomach, salivary gland and larynx expressed OX2R more frequently than other organs.

## Discussion

This study showed the presence of OX2R on several clinical cancer samples including salivary gland, stomach and small

intestine cancer. We demonstrated the existence of OX2R on cell lines as well as on clinical samples using tissue arrays. These results suggest that OX2R is overexpressed in certain types of cancer and may have biological roles in cancer cells. To reveal the functions of OX2R in cancer cells, we conducted additional experiments to confirm the reaction of OX2R-positive cells with a ligand, orexin B. We observed minimal growth promotion of OX2R-positive cancer cells despite increasing ligand



doses (data not shown). Thus, it is suggested that orexin B does not affect the growth of OX2R-expressing cancer cells and that traditional molecular-targeted approaches, such as anti-OX2R antibodies, may not work effectively. Reacting specifically to OX2R and exhibiting toxicity only inside the OX2R-expressing cells may be criteria for drug candidates to target OX2R as a potential mode of action.

Based on insights revealed in this study, we believe that antibodies or ligands conjugated to natural toxins or chemicals, termed immunotoxins or ADCs (21-23), are likely to be an ideal strategy to target receptors such as OX2R. Immunotoxin or ADCs is likely to be an appropriate approach to treat OX2R-positive cancer cells, since antibodies or ligands that bind OX2R themselves are often non-cytotoxic, as previously noted. Immunotoxin is a rationally designed anticancer agent with potent toxins that target cell-surface antigens or receptors on cancer cells. An immunotoxin strategy enables the OX2R antibody to add specific cytotoxicity to cancer cells by fusing the OX2R antibody to toxins. This strategy may also avoid toxicity to the hypothalamus where OX2R is normally expressed with the function of the blood-brain-barrier to maintain homeostasis (24,25). In addition to the direct killing of cancer cells, antibody-dependent cell-mediated cytotoxicity (ADCC) occurs if the antibody to OX2R is conjugated with toxic moiety.

OX2R immunotoxin is an alternative option for patients with resistance to current antibody therapies, since its anti-tumor effect varies from current molecular-targeting drugs. Moreover, dual-specific immunotoxin enables the enhancement of this type of multi-anti-tumor effect. Conjugating the OX2R antibody and kinase inhibitors may elicit both the ADCC and multi-kinase inhibition effects.

In this study, the presence of OX2R on a variety of types of cancer was suggested. However, further studies are required to confirm the presence of OX2R, since the number of clinical samples in the same organs are limited. At present, additional IHC experiments are underway using a larger number of clinical samples to confirm statistical significance of the presence of OX2R and the correlation with stage and cancer type. Therefore, further investigations on OX2R and new immunotoxin/ADC approaches may offer new therapeutic options for cancer patients.

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