

# Possibility of molecular targeting therapy for the treatment of cancer of unknown primary origin by analysis of intracellular signaling molecules

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Received October 19, 2011; Accepted December 9, 2011

DOI: 10.3892/etm.2011.417

**Abstract.** Recently, antibody-mediated epidermal growth factor receptor (EGFR) blockade has become a major research focus, and a number of clinical studies on this new treatment have been started in the field of clinical oncology. This retrospective study investigated the role of KRAS gene mutations and clinical features for possibilities for new therapies in patients with cancer of unknown primary (CUP). We investigated the role of KRAS, PIK3CA and BRAF gene mutations and clinical features for possibilities for new therapies in patients with CUP. Nine patients with metastases from an unknown primary tumor were included in this retrospective study. The KRAS, BRAF and PIK3CA mutational analyses were carried out by means of PCR using genomic DNA for each PCR reaction. The mutation rate in CUP for codon 12 or 13 of the KRAS gene and for PIK3CA was lower than that in colorectal cancer, while the same mutation rate for BRAF was almost the same in the two; this means that the EGFR antibodies can possibly treat CUP.

## Introduction

Cancer of an unknown primary (CUP) is defined as the presence of metastatic cancer in the absence of an identifiable primary tumor site (1). CUP occurs in a heterogeneous group of patients, and subgroups with treatment-responsive diseases that may achieve long-term, disease-free survival exist. Recently, however, the treatment of certain patients with CUP has improved substantially. The identification of treatable patients within this heterogeneous group has been made possible by the development of specialized pathological techniques that aid

in characterizing tumors, and by the recognition that several patients have tumors that respond to chemotherapy.

Recently, antibody-mediated epidermal growth factor receptor (EGFR) blockade has become a major research focus, and much clinical research on this new treatment has been initiated in the field of clinical oncology (2). This retrospective study investigated the role of KRAS gene mutations and clinical features for possibilities for new therapies in patients with CUP.

## Materials and methods

Between October 2004 and December 2009, 9 patients (4 women, 5 men; median age 70.5 years, range 54-87) with metastases from an unknown primary tumor were included in this retrospective study. The definition of CUP syndrome covers patients with histologically confirmed metastatic cancer, when the combination of a complete medical history, accurate physical examination and laboratory tests, and conventional diagnostic imaging procedures (i.e., chest X-ray, abdominal ultrasonograph and, on the basis of suspected primary disease, CT, magnetic resonance imaging, mammography and endoscopic procedures) have failed to identify the primary site. The histological types and sites of metastases are summarized in Table I.

The blood concentrations of CA19-9, CA15-3, CA125 and carcinoembryonic antigen (CEA), and the most commonly used markers – the leukocyte common antigen, cytokeratins, chromogranin and vimentin – were assessed in most cases for histopathological diagnosis.

*DNA extraction from formalin-fixed, paraffin-embedded (FFPE) tumor tissue sections.* Tumor cell-rich areas of H&E-stained sections were marked under a microscope, and tissues were scratched from the same areas of deparaffinized unstained sections. DNA for pieces of the scratched tissue samples was isolated using the QIAamp FFPE Tissue kit (Qiagen KK, Tokyo, Japan).

*Mutational analyses.* KRAS, BRAF and PIK3CA mutational analyses were carried out by means of PCR using 100 ng of genomic DNA for each PCR reaction. All PCR products were directly sequenced using an ABI Prism 377 (Applied

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**Key words:** epidermal growth factor receptor, KRAS, cancer of unknown primary

Table I. Clinicopathological findings.

Case	Gender	Age (years)	PS	Pathological diagnosis	Positive marker	Treatment	Prognosis (months)	Tumor location
1	F	74	1	Malignant mesothelioma	Serum CA125	Resection	4	Abdomen
2	M	80	1	Squamous carcinoma	ND	Resection irradiation	3	Skeletal
3	M	87	2	Adenocarcinoma	Serum CEA	BSC	1	Pleura
4	M	79	3	Undifferentiated carcinoma	ND	BSC	1	Bone marrow
5	F	78	1	Epithelial, maybe adenocarcinoma	AE1/AE3 CAM5.3	Resection	1	Hip joint
6	F	74	3	Maybe adenocarcinoma	CK7	BSC	1	Pleura
7	M	81	4	Squamous carcinoma	ND	BSC	2	Liver
8	M	54	1	Neuroendocrine carcinoma	CD56	CDDP CPT11	13	Liver
9	F	69	1	Adenocarcinoma mucinous type	CK7, CK20 CEA CA19-9 AFP CA125	GEM PTX TS-1	11	Lymph node

PS, physical status; F, female; M, male; ND, not detected; BSC, best supportive care; CDDP, cisplatin; CPT11, irinotecan; GEM, gemcitabine; PTX, paclitaxel.

Table II. Sequence data.

Case	KRAS exon 12	KRAS exon 13	BRAF exon 15V600E	PI3KCA exon 9 E542K	PI3KCA exon 9 E545/D	PI3KCA exon 20 H1047R
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	+	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	+	-	-	-	-	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-

+, positive; -, negative. By analyzing one representative tumor sample in each patient, KRAS mutation in codon 12 was found in one (11%) of the CUP cases. Mutations were not found in codon 13. Of the 11 tumors, 1 (11%) exhibited BRAF mutations at codon 600. PIK3CA mutations were not found.

Biosystems, Foster City, CA, USA) and were then evaluated by means of Sequence Navigator software (Applied Biosystems). Each sequence was carried out at least twice, starting from an independent amplification reaction.

**KRAS.** Exon 1 of the KRAS gene was amplified in order to seek potential mutations on the two foremost codons (12 and 13), which have been reported to be mutated in colorectal cancer (CRC). PCR amplification was carried out using a standard protocol and previously described primers and conditions (2).

**BRAF.** Exons 11 and 15 of the BRAF gene, including the classical mutation V600E, were amplified using previously described conditions and primers (2).

**PI3KCA.** Exons 9 and 20 of the  $\alpha$  polypeptide (the catalytic subunit of the PI3K protein; PI3KCA), which are frequently

mutated in CRC, were amplified using a standard PCR protocol and previously described primers (3).

## Results

**Clinicopathological findings.** Six specimens were adenocarcinomas, two were squamous and one was a neuroendocrine tumor. The histological type of 1 case was not diagnosed despite the use of special immunochemistry. Three cases underwent surgery and 2 were administered chemotherapies. The median survival time was 7 months. The 2 chemotherapy cases had longer survival times, 11 and 13 months, respectively (Table I). Serum CA125 was elevated in 2 cases, and neuroendocrine marker tissue CD56 was positive in 1 case (Table I). Regarding the clinical data in our cases, it was found that in most of them, histological types could be diagnosed using light microscopy, and tissue and markers in the blood served

as auxiliary diagnosis. Markers were particularly useful in diagnosing neuroendocrine tumors.

**KRAS.** By analyzing one representative tumor sample in each patient, KRAS mutation in codon 12 was found in 1 (11%) of the CUP cases (Table II). We could not find any mutations in codon 13.

**BRAF.** Of the 11 tumors, 1 (11%) exhibited BRAF mutations at codon 600 (Table II).

**PIK3CA.** PIK3CA mutations were not found in any tumors (Table II).

## Discussion

Patients with CUP should undergo clinical and pathological evaluation to identify the tumor, followed by appropriate therapy for those who have treatable tumors. Drugs are determined according to the histological type or location of the lesion (4,5). As for antibody-based EGFR-targeted therapy, namely, cetuximab, it is either approved or currently in clinical trial for both squamous cell cancer and adenocarcinoma, and the effects are expected for many histological types of CUP. Also, since gene cluster mutation, which is an effect-prediction marker for this drug, has a significantly lower rate of incidence in CUP than in colon cancer, the effects of these molecular target drugs are greatly expected in CUP.

The *KRAS* gene encodes a small G protein on the EGFR pathway. Cetuximab and other EGFR inhibitors only work on tumors that are not mutated. However, approximately 50% of *KRAS* wild-type patients do not benefit from anti-EGFR treatment (6). To identify the possible causes for this therapy failure, we suggest including mutation analyses of all three EGFR downstream genes as predictive markers in further prospective clinical trials on EGFR-directed therapy of any kind, i.e., not only for CRC. Of note, however, it has been shown that the failure of EGFR antibody therapy may be caused by the activation of mutations of EGFR-dependent signaling molecules. Two main EGFR-dependent signaling pathways are the *KRAS*-*BRAF* and the *PIK3CA*-*PTEN*-*AKT* pathways, which regulate cell proliferation, survival, cell growth, apoptosis resistance, invasion and migration (7). In various clinical studies that included cetuximab or panitumumab in second- or third-line therapy, only patients with wild-type *KRAS* responded to therapy (up to 50% of all patients), whereas patients with tumors exhibiting *KRAS* mutations had a response rate between 0 and 6% (6). Similarly, as shown for *KRAS* mutations, mutations of other EGFR-dependent signaling molecules, such as *BRAF* and *PIK3CA*, may confer resistance to EGFR-specific antibody therapy in CRC as well (8,9). Thus, in a series of 113 patients receiving cetuximab or panitumumab, none of the 11 patients with tumors carrying *BRAF* mutations in a wild-type *KRAS* background responded to therapy. Accordingly, in two other studies, comprising a total of 142 patients with metastatic CRCs, cetuximab failed to

exhibit any significant effect in patients with tumors displaying *PIK3CA* mutations in the background of wild-type *KRAS* (6). Therefore, we analyzed *BRAF* and *PIK3CA* genes in relation to CUP in this study (10).

Considering that in two of the present cases in which chemotherapy was given, the survival time from the initiation of treatment was 11 and 13 months, respectively, it is expected that the combined use of molecular target drugs will further extend prognoses in such cases (11), and application of molecular target drugs to cases in which resection is performed is also expected in the future. In CUP, it may be difficult to expect a complete cure from surgery, and surgery may be limited in its applicability. In the author's own treatment experience, it is difficult to conclude that surgery contributed to prognosis.

In conclusion, the mutation rate in CUP for codon 12 or 13 of the *KRAS* gene, and for *PIK3CA* is lower than in CRC, while the same mutation rate for *BRAF* is almost the same in the two; this means that the EGFR antibody may be effective for CUP. In our study, several cases who were administered chemotherapies showed a relatively good prognosis, and better prognosis will be expected by treatment with an EGFR antibody or a combination of an EGFR antibody and chemotherapy.

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