Gene Expression of Erythropoietin in Hepatocellular Carcinoma
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A 68-year-old man with hepatocellular carcinoma complicated by erythrocytosis showed an increased plasma level of immunoreactive erythropoietin (EPO). Northern blot analysis and RT-PCR (reverse transcriptase and polymerase chain reaction) of EPO mRNA extracted from a surgical specimen indicated high expression of EPO mRNA in the tumor tissue. Histological and immunocytochemical examination showed that the tumor was a hepatocellular carcinoma with predominant immunostaining for EPO. The erythrocytosis improved and the high serum EPO level decreased after resection of the tumor. This is the first demonstration of EPO mRNA expression in hepatocellular carcinoma tissue by RT-PCR.

Key words: Erythrocytosis, PCR, mRNA, tumor

Introduction
Erythropoietin (EPO) is a glycoprotein hormone that is correlated with differentiation and proliferation of erythroid cells. EPO is mainly synthesized in the liver in the fetal period and in the kidney after birth. Its sites of production are thought to be peritubular cells in the kidney and bile duct epithelium in the liver (1, 2). Hypoxia and anemia stimulate EPO synthesis and increase its serum level (3, 4). Erythrocytosis associated with malignant tumors is closely related to a high level of serum EPO. The incidence of hepatocellular carcinomas producing EPO is not high, and the mechanism of production of EPO by tumor cells is still uncertain. Here, we report a case of hepatocellular carcinoma producing EPO associated with erythrocytosis. Studies on the expression of EPO mRNA in the tumor tissue and normal liver tissue of the patient by northern blot analysis and reverse transcriptase and polymerase chain reaction (RT-PCR) are presented, as well as demonstration of the presence of the EPO protein by immunostaining.

Patient and Method

Patient
A 68-year-old Japanese man was admitted to our hospital with abdominal pain. On physical examination, his liver was palpable for 8 cm in the midclavicular line. An initial blood examination showed high levels of tumor markers such as α-fetoprotein (AFP) 34,640 ng/ml (normal range <20 ng/ml), and raised levels of transaminases such as GOT (76 IU/l). Tests for hepatitis virus markers all gave negative results. His ICG (15 min) was 14.9%, RBC 693×10⁶, Hb 20.4 g/dl, Ht 62.0%, WBC 3,800, thrombocyte 17.6×10⁴, serum Fe 58 µg/ml, and ferritin 78 ng/ml. His serum EPO immunoreactivity was 53.0 mU/ml (normal range 8–36 mU/ml), suggesting that the tumor cells were producing EPO. A bone marrow specimen showed mild erythrocytosis, and chromosomal analysis revealed normal 46-XY. Abdominal echo and computerized tomographic (CT) examination revealed the presence of a massive lesion in the right lobe S6-7, which showed irregular enhancement (Fig. 1). Angiography indicated a hypervascular hepatic mass in S6-7 (Fig. 2). By examination of a biopsy specimen, the tumor was identified as a liver cell carcinoma (Edmondson III). The tumor was excised by surgery. Although 520 ml of blood was lost during surgery, blood transfusion was not necessary during operation. By right lobectomy, a tumor of 70×60×55 mm was excised.

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RT-PCR: Total RNA was extracted from the surgical specimen by the GTC (guanidinium thiocyanate)-CsCl (cesium chloride) method. cDNA was synthesized from 5 µg of total RNA with AMV (avian myeloblastosis virus) reverse
transcriptase (RT). The oligonucleotide primers used for PCR of EPO were as follows. The sense primer, which corresponded to the nucleotide sequence (5) of human EPO cDNA (nucleotide 193–213), was 5'-ATATCACTGTCCCAGACACC-3' and the anti-sense primer (nucleotide 464–483) was 5'-AGTGATTGTTCGGAGTGGAG-3'. A volume of 10 μl of cDNA was amplified in a total volume 100 μl. PCR was carried out for 2 min at 55°C, 2 min at 72°C and 2 min at 94°C for 32 cycles for amplification of EPO, 23 for amplification of β-actin. The reaction product was fractionated by electrophoresis on a NuSieve agarose (3%) gel (FMC BioProducts, Rockland, ME).

After electrophoresis, the gel was stained with ethidium bromide.

Northern blot analysis; Poly(A)+mRNA was extracted from 20 μg of total RNA. The total mRNA was separated by electrophoresis in formalin-agarose gel, and transferred to a nylon membrane filter. A 1.2 kb human EPO cDNA was inserted into the ECO RI site of pSP65 vector (5). RNA transcribed by SP6 RNA polymerase and labeled with 32P by the multiprime labelling method was used as the probe. The membrane was prehybridized for 4 hours and hybridized with the RNA probe for 48 hours at 42°C. Then it was washed 3 times with 2×SSC, 0.1% SDS for 20 min each time at room temperature and 3 times with 0.2×SSC, 0.1% SDS for 20 min each time

Fig. 1. Abdominal CT scan demonstrating an irregularly enhanced mass (indicated by arrow) in the right S<sub>7</sub> lobe.

Fig. 2. Angiography revealing a hypervascular mass in S<sub>6</sub>-7.

A. β-actin

B. EPO

![Image of gel showing bands for β-actin and EPO](image_url)

Fig. 3. Amplification of cDNA from the hepatocellular carcinoma and normal liver tissue. A) amplification of β-actin cDNA, B) amplification of erythropoietin (EPO) cDNA. Lane M, marker 174-Hae III digest. Lane 1, negative control (no template); lane 2, normal control (peripheral blood); lane 3, tumor tissue; lane 4, normal liver tissue.
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at 52°C. It was then exposed to preflashed X-ray film for 3 days.

**Immunohistochemical staining**

Tissues were fixed in 10% formalin and embedded in paraffin. Sections of 5 μm thickness were incubated with antiserum to EPO (6) at dilutions of 1:100 to 1:500, and stained by the avidin-biotin peroxidase complex method.

**Results**

**RT-PCR**

The 305-bp regions of β-actin cDNA in normal peripheral blood, tumor tissue and normal liver tissue were amplified equally (Fig. 3A). A strong band of 290-bp of amplified EPO cDNA was detected in tumor cDNA and a weak band in normal tissue of cDNA (Fig. 3B).

**Northern blot analysis**

Studies showed that the EPO probe hybridized to an RNA of 1.2 kb in mRNA extracted from tumor tissue, but not from normal liver tissue (Fig. 4).

**Immunohistochemical staining**

Strong cytoplasmic stainings were observed with antibodies to EPO (Fig. 5) and AFP (data not shown) in tumor tissue, but

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**Fig. 4.** Northern blotting of mRNA in tumor and normal liver tissue. Lane 1, hybridization with β-actin probe in tumor tissue; lane 2, hybridization with erythropoietin (EPO) probe in tumor tissue; lane 3, no hybridization with EPO probe in normal liver tissue.

**Fig. 5.** Immunohistochemical staining of a tumor tissue section (A) and a normal liver tissue section (B) with anti-erythropoietin antibody (x100).
Clinical course

Erythrocytosis and high levels of serum EPO and AFP improved after resection of the tumor (Fig. 6).

Discussion

The complication of erythrocytosis has been observed in malignant tumors such as renal cancers, hepatocellular carcinomas, and cerebral meningioma. In patients with hepatocellular carcinoma, the incidence of erythrocytosis is 3 to 12% (7). However, there have been few studies on gene expression or protein synthesis of EPO in tumor tissues (8–10). In the present case, erythrocytosis with a high serum level of EPO immunoreactivity was observed at the time of admission. Moreover, we confirm higher expression of EPO mRNA in tumor tissue than in normal tissue in surgical specimens of the liver by RT-PCR and northern blot analysis. Immunohistochemical staining also revealed the production of EPO protein in the tumor tissue, but not in normal liver tissue. EPO mRNA is considered to reflect the number of cells producing EPO in the kidney (11). But the mechanism and meaning of EPO production in tumor cells are unknown. In some cases, EPO produced by a tumor seems to have no biological activity (12). This may be why patients with tumors producing tumor EPO are not always complicated with erythrocytosis. Moreover, recently Trimble et al reported that the EPO mRNA obtained from the tumor of a cerebellar hemangioblastoma is smaller than that of normal EPO from hypoxic Hep 3B cells (9). The present case was complicated by erythrocytosis, so the EPO produced by the tumor tissue must have been biologically active. Moreover, the mRNA of tumor EPO was same size as that of normal EPO cDNA by Northern transfer analysis. This is the first demonstration of EPO mRNA expression in hepatocellular carcinoma tissue by RT-PCR. Thus, the tumor cells seemed to produce EPO which is bioactive by some mechanism. Semenza et al observed cell-type specific expression of EPO in the kidney and liver induced by hypoxia, and proposed that multiple regulatory elements control these expressions (13). As no rearrangement of the EPO gene has been reported, it is possible that one of the elements that regulate liver specific expression is changed by malignant transformation of the liver cells.

After resection of the tumor, the present patient’s serum levels of EPO and AFP were normalized and erythrocytosis disappeared. He is now an outpatient of our hospital.

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