Original Article

Expression of neuro-oncological ventral antigen-1 in small-cell lung cancer and its value in pathological diagnosis

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Background: To investigate the expression of neuro-oncological ventral antigen-1 (Nova1) in small-cell lung cancer (SCLC) and its value in clinical pathological diagnosis of SCLCs.

Methods: Nova1 expression in the SCLC cell line H446, lung adenocarcinoma cell line A549, squamous cell carcinoma (SCC) cell line H226 and bronchial epithelial cell line BEAS-2B were examined by RT-PCR. It's expression in paraffin specimens of 100 SCLC cases, 15 specimens of lung adenocarcinoma cases and 15 specimens of lung SCC cases, as well as adjacent normal tissue was determined by immunohistochemistry. The positive rate of Nova1 in SCLC tissue was compared with those of traditional neuroendocrine markers CD56, Syn and CgA.

Results: Nova1 expression in SCLC cell line H446 was significantly higher than that of bronchial epithelial cell line BEAS-2B and lung adenocarcinoma cell line A549 and SCC cell line H226. The positive rate of Nova1 in SCLC tissue was similar to that of CD56 but higher than those of Syn and CgA. Nova1 was neither expressed in the cancer tissue nor in the adjacent normal tissue of the 15 cases of SCC and the 15 cases of adenocarcinoma. In all six cases of combined SCLC, Nova1 was expressed in the SCLC component, whereas markers of SCC or adenocarcinoma were absent.

Conclusions: Nova1 can be used as a novel neuroendocrine marker for pathological diagnosis of SCLC in clinical practice with high sensitivity and specificity, in addition to its role as an independent prognostic marker of overall survival and tumour recurrence, as suggested by previous studies.

Keywords: Nova1; small-cell lung carcinoma; immunohistochemistry

Introduction

Small-cell lung cancer (SCLC) was first reported by WHO in 1926 (1) as a poorly differentiated malignant tumour that is highly proliferative, invasive, metastatic and sensitive to chemotherapeutic drugs but tended to develop drug resistance (2). In 2012, 353,000 people died of lung cancer in Europe (3); SCLC accounted for 10–13% of these deaths, and smoking was a major risk factor. SCLC originates from K cells of the bronchial epithelial mucous gland, which fulfil a neuroendocrine function, and its pathological types include the oat cell, intermediate and mixed types. Under the microscope, tumour cells are spindle-shaped or lymphocyte-like, with few cytoplasm and coarse particles. The cells are often densely integrated into groups, some arranged like parallel fences and shaped like oats. In clinical practice, the characteristic arrangement and cell shape may

be destroyed due to squeezing during sampling, which makes HE sections of SCLC hardly distinguishable from lymphocyte infiltration, poorly differentiated squamous cell carcinoma (SCC), basal cell carcinoma, etc., especially when the sample is taken by fiberoptic bronchoscopy or lung biopsy, which result in a sample with fewer cancer cells. In such situations, it would be extremely important to make full use of immunohistochemical indicators for differential diagnosis. Currently, commonly used markers for SCLC include CD56, Syn and CgA. Compared to Syn and CgA, CD56 is more sensitive to SCLC, but it is also expressed in natural killer cells (NK cells), SCC, adenocarcinoma, mesothelioma, etc., so its specificity is relatively low. Most SCLCs express neuroendocrine markers; nevertheless, up to 10% of SCLC cases are negative for all three neuroendocrine markers (4). In addition, commonly used SCC markers P63, P40, etc., can be expressed in SCLCs, making its diagnosis more difficult. So, it is necessary to find a novel neuroendocrine marker with higher specificity and sensitivity for the diagnosis of SCLCs.

Methods

General information

Paraffin-embedded tissues were collected from the Department of Pathology, Shanghai Oriental Hospital from January 2016 to December 2017, all of which were biopsy or surgical resection specimens. We collected 100 samples of SCLCs, including 84 primary cases and 16 lymph node/liver metastases, and six of them were combined SCLCs. As controls, 15 samples of SCC and 15 samples of adenocarcinoma were also collected. All cases were diagnosed by two senior pathologists according to the 2015 WHO lung cancer histological classification criteria and immunohistochemistry results, and none of the cases were previously treated. All specimens were obtained with informed consent from the patient.

Materials

The major instruments required for the experiment were an RNA extraction kit (MRC, USA), a reverse transcription and qPCR kit, a cDNA synthesiser (TaKaRa, Japan), an amplification instrument (Bio-Rad, USA), a thermocycler (ABI, USA) and an ultra-micro bioassay instrument (Thermo, USA). The antibody used in the experiment was anti-Nova1 (Abcam, UK). The DAB chromogenic kit was

used for immunohistochemistry (Zsbio). Human bronchial epithelial (BEAS-2B), small-cell carcinoma (H446), lung adenocarcinoma (A549) and SCC (H226) cell lines used in the experiments were purchased from the ATCC (Manassas, VA, USA).

Study methods

Cell culture
Cell lines were cultured in RPMI-1640 medium in a 37 ℃ incubator with 5% CO_2.

RNA extraction and quality test
When the cell density reached approximately 80%, total RNA was extracted by adding 1 mL of TRIzol solution. The purity and concentration of the RNA were determined by ultra-micro bioassay absorbance assay, and RNA with an A_{260}/A_{280} between 1.8 and 2.0 was considered sufficiently pure.

RT-PCR
RT-PCR was carried out according to the kit manufacturer's ubstryctuibs, wherein reverse transcription was performed by incubation at 37 ℃ for 15 min and 85 ℃ for 5 s. The cDNA product was used as the template for amplification. The thermocycling parameters were initial denaturation at 95 ℃ for 2 min; 40 cycles of denaturation at 94 ℃ for 15 s and extension at 60 ℃ for 30 s; and a final hold at 4 ℃ The melting curve was drawn for quality control, and quantification was carried out by using the $2^{-\Delta\Delta Ct}$ method. Three duplicate wells were set in each group, and the experiment was repeated three times. GAPDH was selected as the internal control. The corresponding PCR primers were as follows: NOVA1, forward, 5'-GGTCTCAGCCAAGCAGCAGCAA-3' and reverse, 5'-TTGCAGCAGTAGCAGCAGCCAG-3'; GAPDH, forward, 5'-TGGATCTGACATGCCGCCTGGA-3' and reverse, 5'-AGGTCCACCA CCCTGTTGCTGT-3'.

Immunohistochemistry
The above-mentioned tissue blocks were fixed with 4% formaldehyde, conventionally dehydrated before embedding in paraffin and sliced into 4-μm sections Immunohistochemical of neuro-oncological ventral antigen-1 (Nova1), P63, P40, CD56, synaptophysin (Syn) and chromogranin A (CgA) were performed using the EnVision two-step method on the Dako Omnis automated

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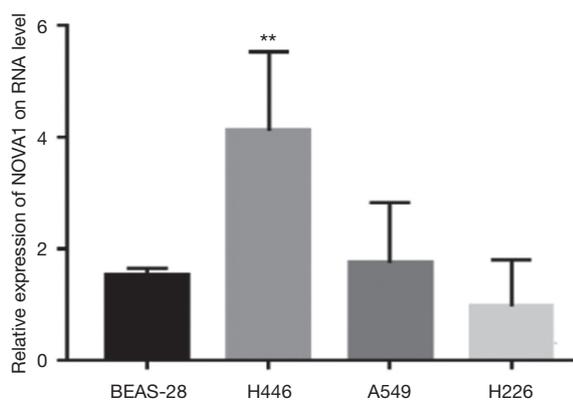
Figure 1 Nova1 mRNA was highly expressed in the SCLC H446 cell line. Note: BEAS-2B (normal bronchial epithelial cell line), H446 (SCLC cell line), A549 (lung adenocarcinoma cell line), H226 (squamous cell carcinoma cell line), **, P<0.05.

immunohistochemical stainer. All target antigens were prepared with high- pressure sodium citrate buffer at pH 6.0. PBS was used in place of the primary antibody for a blank control, and already known positive cases were used as positive controls. The specific steps were carried out according to the kit manufacturer's instructions. In immunohistochemical staining, Nova1 was localised in the nucleus, Syn and CgA were localised in the cytoplasm, CD56 was localised in the cell membrane and/or cytoplasm, and P63 and P40 were localised in the nucleus. For Nova1 staining, <5% of cells stained under a 400× field was considered negative expression, 5–50% was considered low expression and >50% was considered high expression.

Statistical analysis

Experimental data were analysed using SPSS 22.0 analysis software. Measurement data were represented as mean ± standard deviation; comparison between two groups was performed with a Student's *t*-test. Count data were analysed by employing a Chi-square test. Results were considered statistically significant at P<0.05.

Results

Expression of *Nova1* gene at cellular level Bioinformatics analysis using the Oncomine database suggested that the expression level of Nova1 in SCLC was significantly higher than that in normal lung tissue, adenocarcinoma SCC and large-cell carcinoma. In our research, RT-PCR results

demonstrated that the expression of Nova1 mRNA in SCLC H446 cells was higher than that in normal BEAS-2B bronchial epithelial cells and lung adenocarcinoma A549 or H226 SCC cells (*Figure 1*).

Nova1 expression in tissue samples

Expression of Nova1 in SCLC

The positive rate of Nova1 in SCLC was 88% (*Figure 2*). Nova1 expression in primary and metastatic SCLC tissues was significantly higher than that in lung SCC and lung adenocarcinoma tissues. Compared to lymph node/liver metastases, Nova1 expression was significantly higher in primary SCLC (χ^2=6.684, P=0.001), as shown in *Table 1*.

Expression of Nova1 in NSCLC

Nova1 was neither expressed in the cancer tissue nor in the adjacent normal tissue of the 15 cases of SCC and the 15 cases of adenocarcinoma. Six cases of combined SCLC were included in the sample, including three cases of SCLC complicated by SCC and three cases of SCLC complicated by adenocarcinoma. Nova1 expression was detected in the SCLC components of all six cases but absent in the SCC or adenocarcinoma component, as shown in *Figure 3*.

Positive rates of Nova1, CD56, CgA, SYN, P63 and P40 in SCLC tissues

Positive rates of Nova1, CD56, SYN, CgA, P63 and P40 in SCLC were 88% (88/100), 92% (92/100), 75% (75/100), 79% (79/100), 13% (13/100) and 7% (7/100), respectively. The rate of Nova1 positivity was not significantly different from that of CD56 (χ^2=0.889, P=0.336) but was significantly higher than that of Syn and CgA (χ^2=5.604, P=0.018 and χ^2=2.940, P=0.086). The commonly used SCC markers P63 and P40 had positivity rates of 13% and 7% in the SCLC samples, respectively. As shown in *Table 2*.

Discussion

Expression of Nova1 in SCLC specimens

Nova1 is a central nervous system-specific RNA-binding protein. As a self-antigen of the nervous system, it was first identified in patients with paraneoplastic opsoclonus-myoclonus ataxia (POMA) (5,6). In recent years, it has been found that Nova1 plays an important role in the development of lymphoma, multiple myeloma, gastric cancer, melanoma, astrocytoma and oligodendroglioma
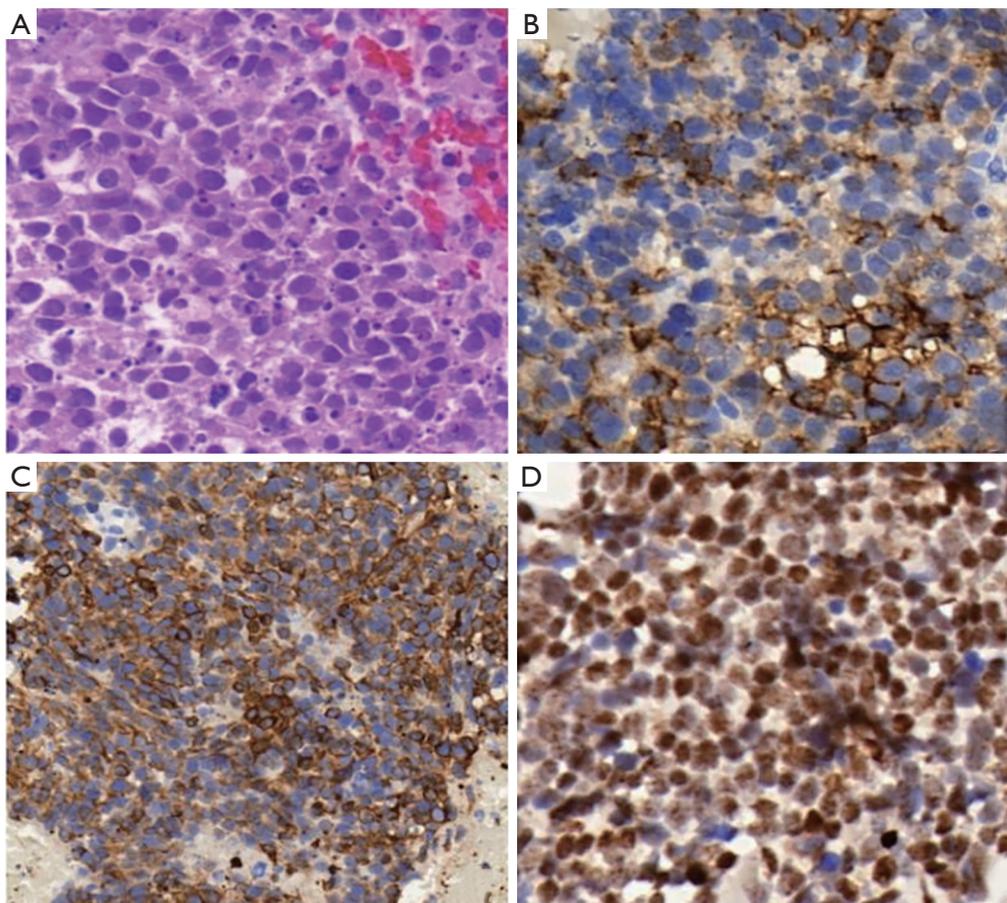
Figure 2 Expression of CD56, CKpan and Nova1 in SCLC. (A) SCLC (HE, ×400), (B) expression of CD56 in SCLC (IHC, ×400), (C) expression of CKpan in SCLC (IHC, ×200), (D) expression of Nova1 in SCLC (IHC, ×400). SCLC, small-cell lung cancer.

Table 1 Comparison of Nova1 expression between small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) or their lymph node/liver metastases

Group	Tissue	Nova1 expression			
		Samples	Negative	Low	High
SCLC	Primary lesion	84	7	16	61
	Lymph node/liver metastasis	16	5	4	7
NSCLC	Primary lesion	25	25	0	0
	Lymph node/liver metastasis	5	5	0	0

(7-10). Zhang *et al.* (11,12) showed that expression of Nova1 in hepatocellular carcinoma Huh7 and SMMC-7721 cell lines was significantly higher than that in normal hepatocyte cell lines. Moreover, down-regulation of Nova1 expression resulted in decreased proliferation and invasion of Huh7 cells, whereas Nova1 overexpression enhanced proliferation and invasion in SMMC-7721 cells. Similar results were also obtained in a nude mouse model of primary liver cancer. They further compared Nova1 expression in cancerous tissue and adjacent normal tissue of 91 patients with hepatocellular carcinoma and found that Nova1 expression was higher in the cancerous tissue,
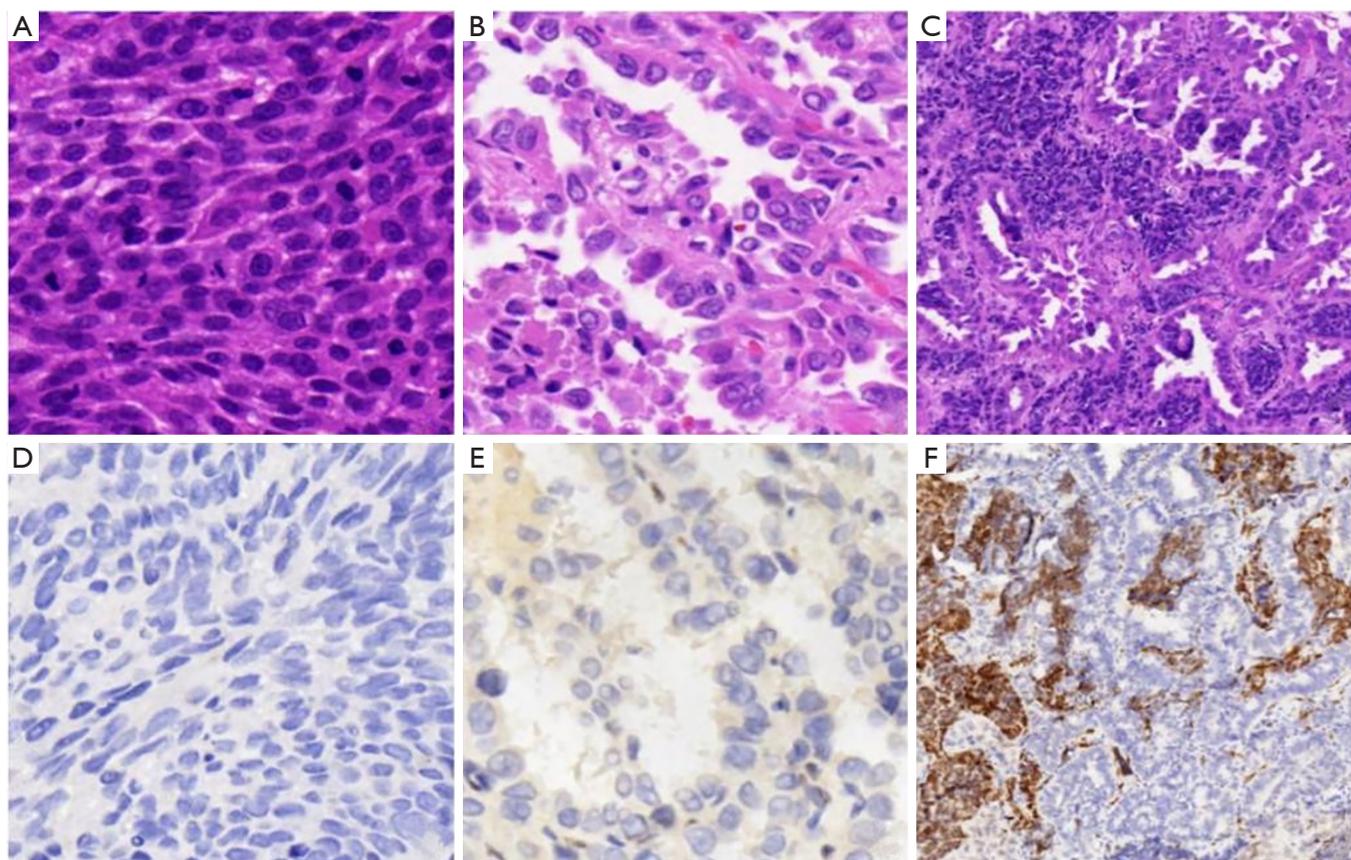
Figure 3 Expression of Nova1 in NSCLC. (A) Squamous cell carcinoma (HE, ×400), (B) adenocarcinoma (HE, ×400), (C) SCLC complicated with adenocarcinoma (HE, ×100), (D) nova1 was negative in squamous cell carcinoma (IHC, ×400), (E) nova1 expression was negative in adenocarcinoma (IHC, × 400), (F) Nova1 expression was strongly positive in small-cell lung carcinoma cells, which showed a pattern of nucleocytoplasmic staining, but expression was negative in adenocarcinoma (IHC, × 100). SCLC, small-cell lung cancer.

Table 2 Comparison of Nova1, CD56, SYN, CgA, P63 and P40 expression in 100 samples of small-cell carcinoma

Marker	Negative	Low	High
Nova1	12	20	68
CD56	8	29	63
SYN	25	31	44
CgA	21	24	55
P63	87	3	10
P40	93	2	5

and higher Nova1 expression was correlated with poorer prognosis, representing an independent risk factor for patient survival and tumour recurrence. Yu *et al.* (13) used immunohistochemistry to examine three cases of malignant melanoma and two cases of intradermal naevus and found that all the malignant melanoma specimens expressed Nova1, while all the intradermal naevus specimens were Nova1-negative. Suppression of Nova1 expression in melanoma cell lines inhibited proliferation, metastasis and invasion in melanoma A375 and A385 cells, while overexpressing Nova1 had the opposite effect. Zhi *et al.* (14) demonstrated that Nova1 is regulated by miR-181b-5p in astrocytoma, and elevated Nova1 expression in this tumour promotes cell proliferation and invasion and inhibits apoptosis; when miR-181b-5p was overexpressed in astrocytoma, Noval expression was significantly downregulated, cell proliferation and invasion were inhibited and the apoptosis rate was significantly increased. Yet, Kim *et al.* (8) demonstrated that Nova1 was significantly

downregulated in gastric cancer and gastric cancer cell lines, and lower Nova1 expression was closely related to poorer prognosis in patients with gastric cancer. So far, there has been no report about Nova1 expression in SCLC. In this study, it was found that Nova1expression was significantly higher in the SCLC cell line H446 than in the bronchial epithelial cell line BEAS-2B and lung adenocarcinoma cell lines A549 and H1299.Immunohistochemistry analysis also demonstrated that Nova1 was expressed in up to 88% of SCLC specimens.

The role of Nova1 in pathological diagnosis of SCLC

Traditional neuroendocrine markers include CD56, SYN, CgA, and the like; among them, CD56 is mainly expressed in relatively undifferentiated neuroendocrine tumours, which is helpful for the diagnosis of SCLC. Kontogianni *et al.* (15) and Yeh and Chou (16) reported that 97–100% of SCLCs express CD56, and in this group, the rate of CD56 positivity was 92%. However, CD56 is also expressed in NK cells, SCC, adenocarcinoma and mesothelioma, showing poor specificity. Syn has relatively low sensitivity for SCLCs, and CgA is often not expressed in relatively undifferentiated SCLCs, which contain fewer cytoplasmic secretory granules (16,17). In recent years, scholars have found that the commonly used squamous cell marker p63 is present in 30–51% of lung adenocarcinomas (18) and in 0–76% of SCLCs (18-20). In this study, the rate of p63 positivity was 13%, similar to the value of 14% obtained by Hamanaka *et al.* (21). P40 is highly sensitive and specific for SCC and is thus considered the best marker for this cancer. However, Nakajima's research shows that although p40 was not diffusely expressed in any of the SCLC cases, the rate of P40 positivity in small-cell carcinoma can be as high as 34.1% (22). The rate of P40 positivity in this group of SCLC patients was 7%, which was higher than that reported by Tatsumori *et al.* 5% (18). At present, no studies on Nova1 expression in SCLC have been reported. In this study, the rate of Nova1 positivity in SCLC patients was 88%, as determined by the presence of brown staining in immunohistochemistry, and 72.3% of cases showed high levels of Nova1 expression. Nova1 was not expressed in non-neuroendocrine tumours such as SCC and adenocarcinoma, suggesting that Nova1 is highly specific for SCLC. In addition, SCLC is a highly malignant tumour with extremely high mortality and poor prognosis, mostly due to its invasive tumour growth and metastasis. A number of *in vitro* studies have shown that abnormal activation of

Nova1 can promote cell differentiation and proliferation, inhibit cell apoptosis and promote tumour cell metastasis, suggesting that Nova1 expression is negatively correlated with prognosis and can be used as an independent prognostic marker. It should be noted that although Nova1 is also highly expressed in hepatocellular carcinoma, malignant melanoma, astrocytoma and oligodendroglioma, they can be differentiated from SCLC in terms of the primary tumour site, histomorphology and other immune markers, so it does not undermine the clinical value of Nova1 as a specific marker for SCLC.

In summary, RT-PCR showed that mRNA levels of Nova1 were significantly increased in SCLC cells relative to normal bronchial epithelial cells, adenocarcinoma cells and SCC cells. Further immunohistochemical examination of paraffin- embedded specimens 100 SCLCs confirmed that Nova1 can be used as a novel neuroendocrine marker for clinical pathological diagnosis of SCLC. Moreover, compared with traditional neuroendocrine markers, Nova1 has high sensitivity and specificity, in addition to its role as a predictor of recurrence and prognosis.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi. org/10.21037/tcr.2019.12.99). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This research was approved by the Medical Ethics Committee of Shanghai East Hospital, Tongji University School of Medicine. All specimens were obtained with informed consent from the patient.

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