

# CDH11 Expression is Associated with Survival in Patients with Osteosarcoma

Go Nakajima<sup>1\*</sup>, Ana Patiño-Garcia<sup>2\*</sup>, Skjalg Bruheim<sup>3</sup>, Yaguang Xi<sup>1</sup>, Mikel San Julian<sup>2</sup>, Fernando Lecanda<sup>4</sup>, Luis Sierrasesumaga<sup>2</sup>, Christoph Müller<sup>3</sup>, Oystein Fodstad<sup>1,3</sup> and Jingfang Ju<sup>1</sup>

<sup>1</sup>Cancer Genomics Laboratory, Mitchell Cancer Institute-USA, Mobile, AL, U.S.A.

<sup>2</sup>University Clinic of Navarra, Pamplona, Spain

<sup>3</sup>Norwegian Radium Hospital, Oslo, Norway

<sup>4</sup>Laboratory of Adhesion and Metastasis, Cancer for Medical Applied Medicine, CIMA, Pamplona, Spain

\*Both authors contributed equally to this study.

Correspondence to: Jingfang Ju, Ph.D., Head, Cancer Genomics Laboratory, Assistant Professor of Pharmacology and Medicine, Mitchell Cancer Institute, MSB2316, 307 N. University Blvd., Mobile, AL 36688, U.S.A. Tel: +1 251 460 7393, Fax: +1 251 460 6994, e-mail: [jjju@usouthal.edu](mailto:jjju@usouthal.edu)

## ABSTRACT

Previous studies have shown that cadherin-11 (CDH11) may be involved in the metastatic process of osteosarcoma. The correlation of the expression levels of CDH11 in osteosarcoma samples with the risk of disease progression and metastasis was examined. Real time qRT-PCR was used to quantify CDH11 expression in a set of newly established osteosarcoma cell lines, 11 primaries and five metastases, compared to the levels in 12 normal osteoblast cell lines established from healthy bone, and also in a set of 10 snap-frozen osteosarcoma samples. In all cases long term clinical follow-up data was available. The CDH11 expression level decreased gradually from the osteoblast to the primary cell lines ( $p=0.2184$ ) and further to those established from the tumor metastases ( $p=0.0275$ ). Importantly, the level of CDH11 expression correlated significantly ( $p=0.01$ ) with patient survival (Kaplan-Meier survival analysis) in both sample sets ( $p=0.0128$  for the cell lines,  $p=0.0492$  for the biopsies). In conclusion, the results indicate that CDH11 may be useful as a prognostic marker of disease progression and survival in osteosarcoma.

## KEY WORDS

Osteosarcoma, biomarker, CDH11, prognosis.

## **INTRODUCTION**

Osteosarcoma is a common malignant bone-forming mesenchymal tumor, commonly arising in the extremities of adolescents and young adults. Although advances in chemotherapy have improved the survival rates during the last three decades (1, 2), approximately 40% of the patients do not survive, mainly due to lung metastases (3). Traditional prognostic indicators, such as age, gender, primary tumor location, disease-free interval, tumor doubling time, bilaterality, respectability and number of detectable pulmonary metastases (4, 5) have had limited success in identifying those patients that need aggressive chemotherapy and those that do not. Since at present all patients receive pre- and post-operative chemotherapy, biomarkers that could distinguish between patients that would benefit from the current toxic chemotherapy and those that do not need chemotherapy or those that should receive other treatment alternatives instead are needed (6). Currently, however, few biomarkers that can be used as predictors for patient outcome are available. Several recent studies have identified genetic alterations and/or gene expression abnormalities that may be used as targets or biomarkers in osteosarcoma (7-13), and studies using cell lines or tumor tissue biopsies have related tumor gene expression profiles to response to a specific chemotherapy (10, 12, 14-16). However, few if any of them have made any real impact in the clinical setting.

Cadherins are important cell surface molecules for cell-cell communication and signaling through catenin (17-19), and have been associated with tumor progression in several types of cancer (18, 20, 21). It is known that cadherins modulate calcium-dependent cell adhesion, cell aggregation and migration (22, 23). Alterations in cadherins and catenins are known to contribute to tumor initiation, progression and metastasis by impacting the Wnt signaling pathway, which is a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions (17-19). Both  $\beta$ - and  $\gamma$ -catenin are involved in the oncogenic process of many tumor types (24, 25). Over 20 types of cadherins have been discovered and characterized (22, 26), including cadherin11 (CDH11) which is identical to OB-cadherin and has approximately 60% amino acid homology with N-cadherin (27). Based on the putative important function of CDH11 in osteosarcoma, the expression of CDH11 might be useful as a prognostic marker in osteosarcoma.

In this study, the potential prognostic value of CDH11 was evaluated using real time qRT-PCR analysis of cultured cells derived from primary and metastatic osteosarcoma tumors and paired normal bone, as well as on snap-frozen osteosarcoma samples.

## **MATERIALS AND METHODS**

### **Samples**

A set of osteosarcoma cell lines derived from 11 untreated primary and five metastatic tumors and 12 paired osteoblastic cell lines established from adjacent normal bone tissue from patients treated and followed up at the University Clinic of Navarra, Pamplona, Spain, were studied. Needle biopsy specimens were disaggregated and cultured in  $\alpha$ MEM (Invitrogen S.A., Barcelona, Spain) supplemented with 10% FCS and penicillin/streptomycin. If necessary, samples were first treated with a collagenase

solution for 1 hour at 37°C with agitation, and the sample was filtered through 70 µm filters. The cells were then incubated at 37°C in a 5% CO<sub>2</sub> atmosphere and the medium was replaced every three days. After the cells from the tissue explants covered the plastic surface, they were trypsinized and passaged. The experiments were performed with either first or second passages, in all cases, the cells were 90% confluent and in the logarithmic growth phase. The clinical data for these patients is summarized in Table I.

In a second study set, 10 untreated osteosarcoma biopsy samples from patients treated at the Norwegian Radium Hospital, Oslo, Norway, were used and the clinical data for these is shown in Table II.

### **RNA isolation**

The total RNA was isolated using TRIzol<sup>®</sup> (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. In brief, 1 ml TRIzol was added and mixed by vortexing at room temperature. Two hundred µl 1-bromo-3-chloropropane (BCP) was added and mixed by vortexing, and then the samples were centrifuged at 4°C for 10 min at 14,000 rpm.

The upper aqueous phase was transferred to a new microcentrifuge tube. Then, equal volume 100% isopropanol was added to the tube which contained the upper aqueous phase and mixed by vortexing, and the total RNA was precipitated at -20°C for 1 hour. The samples were centrifuged for 10 min at 14,000 rpm at 4°C. After removal of the supernatant, 500 µl 75% ethanol was added. The samples were centrifuged for 10 min at 14,000 rpm at 4°C and the RNA pellet was air-dried and eluted with nuclease-free water.

### **Reverse transcription (RT)**

Single-stranded cDNA synthesis was performed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. In brief, a total reaction volume of 100 µL was made including 1 µg RNA template, 10 µl 10x reverse transcription buffer, 4 µl 25x dNTPs, 10 µl 10x random primers, 5 µl 50U/µl MultiScribe<sup>™</sup> reverse transcriptase and 1 µl RNasin<sup>®</sup> Plus RNase inhibitor (Promega). The RT reaction was performed at 25°C for 10 min and then followed by 120 min at 37°C.

### **Real-time PCR**

Quantitative real-time PCR (qRT-PCR) was carried out using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Primer/probe pairs for CDH11 (CDH11; Assay ID Hs00156438\_m1) and internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Assay ID Hs99999905\_m1) were obtained from TaqMan<sup>®</sup> Gene Expression Assays (Applied Biosystems, Foster City, CA). A total reaction volume of 25 µL was made for each well, including the cDNA from each sample, TaqMan<sup>®</sup> Universal PCR Master Mix (Applied Biosystems) and each target primer/probe. The qRT-PCR analysis was performed in triplicate for each sample. PCR

was performed at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

### **Data analysis**

The threshold cycle ( $C_T$ ) value for each gene was determined by SDS software v1.2 (Applied Biosystems). Delta- $C_T$  ( $\Delta C_T$ ) value, which is the difference between the  $C_T$  value of the target gene and the  $C_T$  value of the endogenous control gene was also calculated by the same software. Delta- $\Delta C_T$  ( $\Delta\Delta C_T$ ), which is the difference in the  $\Delta C_T$  value for each sample and the highest  $\Delta C_T$  value as a calibrator, was calculated. The  $2^{-\Delta\Delta C_T}$  number was used for relative quantification.

The statistical analyses were performed using MedCalc<sup>®</sup> for Windows, version 9.2.0.1 (MedCalc software, Mariakerke, Belgium). The expression level of CDH11 was compared among the different sample types using the Mann-Whitney test. The log-rank test for Kaplan-Meier survival was used to assess the association between CDH11 expression level and survival. Statistical significance was set at p-value 0.05 or lower.

### **RESULTS**

Real time qRT-PCR analysis indicated a tendency, although not significant ( $p=0.22$ ), of decreased expression levels from normal osteoblastic cell cultures to primary osteosarcoma cell lines. Notably, comparing the CDH11 expression in the cell lines derived from the 11 primary and the five metastases showed a significant reduction in CDH11 expression level ( $p=0.0275$ ) in the latter cell lines (Figure 1).

The Kaplan-Meier survival plot revealed that the patients with higher CDH11 expression in their primary tumor cell lines had significantly longer survival time compared to those with lower CDH11 expression ( $p=0.01$ ; log-rank test) (Figure 2). These results were further strengthened by the data for the osteosarcoma snap-frozen specimens, as also in this sample set also the group with higher CDH11 expression had a longer survival time than the group with lower CDH11 expression ( $p=0.04$ ; log-rank test) (Figure 3).

### **DISCUSSION**

In this study, a strong and significant association was found between CDH11 expression and patient survival in both the group of paired osteosarcoma and osteoblast cell lines derived from samples of diseased and normal bone and the set consisted of snap-frozen biopsy specimens from untreated osteosarcoma patients. The significant association with survival was seen despite the relatively small sample size; further validation will require a large number of biopsy samples also obtained preferably before the beginning of neoadjuvant chemotherapy. The fact that osteosarcoma is a relatively rare disease emphasizes the need for multi-institutional collaboration to identify and validate new biomarkers.

Our results also showed a tendency of reduced CDH11 expression in the cell lines from primary tumors compared to the normal osteoblast cultures, and furthermore that the expression level was significantly decreased in cell lines from metastases compared to the primary tumors (Figure 1). This supported previous data indicating that the loss of CDH11 may be a key event in osteosarcoma metastasis (28). Conceivably, the decrease in CDH11 expression may be associated with alterations in catenin expression and tumor invasion. Several studies have suggested that the loss or decrease of CDH11 expression is an important event in tumor metastasis including retinoblastoma and osteosarcoma (20, 28-30). CDH11 is highly expressed in osteoblastic cell lines and plays an important role in osteoblast differentiation (31-33). However, Kashima et al. found anomalous expression of CDH11 in osteosarcoma, but overexpression of the gene suppressed pulmonary metastasis (34). In the case of osteosarcoma, it seems that loss or decreased CDH11 expression, much like the function of E-cadherin in many tumor types, contributes to tumor invasion (21, 35-40), and it has been shown that osteosarcoma metastasis can be prevented by restoration of the CDH11 using a mouse model (34). The decrease or loss of cell differentiation potential associated with the reduction of CDH11 expression may contribute to the oncogenic capacity of osteosarcoma and the sensitivity to chemotherapy (41). Furthermore, a recent report has demonstrated the role of CDH11 in the inflammation process in arthritis suggesting a potential link between inflammation, tumor invasion and metastasis in osteosarcoma (42).

In summary, a reduced level of CDH11 expression is inversely correlated with osteosarcoma survival, as studied in samples obtained from two independent sources of tumor material with available clinical follow-up data. The loss of CDH11 may be a significant event in the progression and metastasis of osteosarcoma, and the results provide a basis to pursue future studies with a larger number of patient samples obtainable by a joint effort of different cancer centers.

## **ACKNOWLEDGEMENTS**

We appreciate the critical reviews and Gunhild M. Malandsmo and Dr. Hege Oma Ohnstad for updating the clinical information on the Norwegian patients. This study was supported by the Mitchell Cancer Institute start-up fund (J. Ju), the Spanish Ministry of Health, Instituto de Salud Carlos III, FIS, Ref. PI040010 and Gobierno de Navarra, Department of Health, Ref. 17/2004 (A.P-G., L.S., M.S.J., F.L.), and The Norwegian Cancer Society.

## **REFERENCES**

- 1) Bramwell VH, Santoro A, Rouesse J, Mouridsen H, Steward W, Van Oosterom A, Somers R, Buesa J, Mulder J, Schutte J et al: Review of the clinical trials activity of the Soft Tissue and Bone Sarcoma Group of the European Organization for Research and Treatment of Cancer. *Semin Surg Oncol* 4: 45-52, 1988.
- 2) Meyers PA and Gorlick R: Osteosarcoma. *Pediatr Clin North Am* 44: 973-989, 1997.

- 3) Ward WG, Mikaelian K, Dorey F, Mirra JM, Sassoon A, Holmes EC, Eilber FR and Eckardt JJ: Pulmonary metastases of stage IIB extremity osteosarcoma and subsequent pulmonary metastases. *J Clin Oncol* 12: 1849-1858, 1994.
- 4) Mialou V, Philip T, Kalifa C, Perol D, Gentet JC, Marec-Berard P, Pacquement H, Chastagner P, Defaschelles AS and Hartmann O: Metastatic osteosarcoma at diagnosis: prognostic factors and long-term outcome – the French pediatric experience. *Cancer* 104: 1100-1109, 2005.
- 5) Tsuchiya H, Kanazawa Y, Abdel-Wanis ME, Asada N, Abe S, Isu K, Sugita T and Tomita K: Effect of timing of pulmonary metastases identification on prognosis of patients with osteosarcoma: the Japanese Musculoskeletal Oncology Group study. *J Clin Oncol* 20: 3470-3477, 2002.
- 6) Bruland OS and Pihl A: On the current management of osteosarcoma. A critical evaluation and a proposal for a modified treatment strategy. *Eur J Cancer* 33: 1725-1731, 1997.
- 7) Hoang BH, Dyke JP, Koutcher JA, Huvos AG, Mizobuchi H, Mazza BA, Gorlick R and Healey JH: VEGF expression in osteosarcoma correlates with vascular permeability by dynamic MRI. *Clin Orthop Relat Res* pp. 32-38, 2004.
- 8) Laverdiere C, Hoang BH, Yang R, Sowers R, Qin J, Meyers PA, Huvos AG, Healey JH and Gorlick R: Messenger RNA expression levels of CXCR4 correlate with metastatic behavior and outcome in patients with osteosarcoma. *Clin Cancer Res* 11: 2561-2567, 2005.
- 9) Mintz MB, Sowers R, Brown KM, Hilmer SC, Mazza B, Huvos AG, Meyers PA, Lafleur B, McDonough WS, Henry MM, Ramsey KE, Antonescu CR, Chen W, Healey JH, Daluski A, Berens ME, Macdonald TJ, Gorlick R and Stephan DA: An expression signature classifies chemotherapy-resistant pediatric osteosarcoma. *Cancer Res* 65: 1748-1754, 2005.
- 10) Hayden JB and Hoang BH: Osteosarcoma: basic science and clinical implications. *Orthop Clin North Am* 37: 1-7, 2006.
- 11) Hennig G, Behrens J, Truss M, Frisch S, Reichmann E and Birchmeier W: Progression of carcinoma cells is associated with alterations in chromatin structure and factor binding at the E-cadherin promoter in vivo. *Oncogene* 11: 475-484, 1995.
- 12) Osaka E, Suzuki T, Osaka S, Yoshida Y, Sugita H, Asami S, Tabata K, Hemmi A, Sugitani M, Nemoto N and Ryu J: Survivin as a prognostic factor for osteosarcoma patients. *Acta Histochem Cytochem* 39: 95-100, 2006.
- 13) Ricci C, Landuzzi L, Rossi I, De Giovanni C, Nicoletti G, Astolfi A, Pupa S, Menard S, Scotlandi K, Nanni P and Lollini PL: Expression of HER/erbB family of receptor tyrosine kinases and induction of differentiation by glial growth factor 2 in human rhabdomyosarcoma cells. *Int J Cancer* 87: 29-36, 2000.
- 14) Forus A, Weghuis DO, Smeets D, Fodstad O, Myklebost O and Geurts van Kessel A: Comparative genomic hybridization analysis of human sarcomas: II. Identification of novel amplicons at 6p and 17p in osteosarcomas. *Genes Chromosomes Cancer* 14: 15-21, 1995.
- 15) Goeman JJ, Oosting J, Cleton-Jansen AM, Anninga JK and van Houwelingen HC: Testing association of a pathway with survival using gene expression data. *Bioinformatics* 21: 1950-1957, 2005.
- 16) Osaka E, Suzuki T, Osaka S, Yoshida Y, Sugita H, Asami S, Tabata K, Sugitani M, Nemoto N and Ryu J: Survivin expression levels as independent predictors of survival for osteosarcoma patients. *J Orthop Res* 25: 116-121, 2007.

- 17) Jankowski JA, Bruton R, Shepherd N and Sanders DS: Cadherin and catenin biology represent a global mechanism for epithelial cancer progression. *Mol Pathol* 50: 289-290, 1997.
- 18) Nakamura E, Sugihara H, Bamba M and Hattori T: Dynamic alteration of the E-cadherin/catenin complex during cell differentiation and invasion of undifferentiated-type gastric carcinomas. *J Pathol* 205: 349-358, 2005.
- 19) Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ and Schalken JA: Cadherin switching in human prostate cancer progression. *Cancer Res* 60: 3650-3654, 2000.
- 20) Pishvaian MJ, Feltes CM, Thompson P, Bussemakers MJ, Schalken JA and Byers SW: Cadherin-11 is expressed in invasive breast cancer cell lines. *Cancer Res* 59: 947-952, 1999.
- 21) Oka H, Shiozaki H, Kobayashi K, Inoue M, Tahara H, Kobayashi T, Takatsuka Y, Matsuyoshi N, Hirano S, Takeichi M et al: Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res* 53: 1696-1701, 1993.
- 22) Takeichi M: Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* 7: 619-627, 1995.
- 23) Vleminckx K, Vakaet L Jr, Mareel M, Fiers W and van Roy F: Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 66: 107-119, 1991.
- 24) Soler AP, Johnson KR, Wheelock MJ and Knudsen KA: Rhabdomyosarcoma-derived cell lines exhibit aberrant expression of the cell-cell adhesion molecules N-CAM, N-cadherin, and cadherin-associated proteins. *Exp Cell Res* 208: 84-93, 1993.
- 25) Fukata M and Kaibuchi K: Rho-family GTPases in cadherin-mediated cell-cell adhesion. *Nat Rev Mol Cell Biol* 2: 887-897, 2001.
- 26) Nollet F, Kools P and van Roy F: Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J Mol Biol* 299: 551-572, 2000.
- 27) Okazaki M, Takeshita S, Kawai S, Kikuno R, Tsujimura A, Kudo A and Amann E: Molecular cloning and characterization of OB-cadherin, a new member of cadherin family expressed in osteoblasts. *J Biol Chem* 269: 12092-12098, 1994.
- 28) Kashima T, Kawaguchi J, Takeshita S, Kuroda M, Takanashi M, Horiuchi H, Imamura T, Ishikawa Y, Ishida T, Mori S, Machinami R and Kudo A: Anomalous cadherin expression in osteosarcoma. Possible relationships to metastasis and morphogenesis. *Am J Pathol* 155: 1549-1555, 1999.
- 29) Kawaguchi J, Takeshita S, Kashima T, Imai T, Machinami R and Kudo A: Expression and function of the splice variant of the human cadherin-11 gene in subordination to intact cadherin-11. *J Bone Miner Res* 14: 764-775, 1999.
- 30) Marchong MN, Chen D, Corson TW, Lee C, Harmandayan M, Bowles E, Chen N and Gallie BL: Minimal 16q genomic loss implicates cadherin-11 in retinoblastoma. *Mol Cancer Res* 2: 495-503, 2004.
- 31) Rieger-Christ KM, Cain JW, Braasch JW, Dugan JM, Silverman ML, Bouyounes B, Libertino JA and Summerhayes IC: Expression of classic cadherins type I in urothelial neoplastic progression. *Hum Pathol* 32: 18-23, 2001.
- 32) Voura EB, Sandig M and Siu CH: Cell-cell interactions during transendothelial migration of tumor cells. *Microsc Res Tech* 43: 265-275, 1998.

- 33) Cheng SL, Lecanda F, Davidson MK, Warlow PM, Zhang SF, Zhang L, Suzuki S, St John T and Civitelli R: Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. *J Bone Miner Res* 13: 633-644, 1998.
- 34) Kashima T, Nakamura K, Kawaguchi J, Takanashi M, Ishida T, Aburatani H, Kudo A, Fukayama M and Grigoriadis AE: Overexpression of cadherins suppresses pulmonary metastasis of osteosarcoma in vivo. *Int J Cancer* 104: 147-154, 2003.
- 35) Schipper JH, Frixen UH, Behrens J, Unger A, Jahnke K and Birchmeier W: E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. *Cancer Res* 51: 6328-6337, 1991.
- 36) Bringuier PP, Umbas R, Schaafsma HE, Karthaus HF, Debruyne FM and Schalken JA: Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. *Cancer Res* 53: 3241-3245, 1993.
- 37) Dorudi S, Sheffield JP, Poulson R, Northover JM and Hart IR: E-cadherin expression in colorectal cancer. An immunocyto-chemical and in situ hybridization study. *Am J Pathol* 142: 981-986, 1993.
- 38) Mayer B, Johnson JP, Leitl F, Jauch KW, Heiss MM, Schildberg FW, Birchmeier W and Funke I: E-cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Res* 53: 1690-1695, 1993.
- 39) Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HF, Oosterhof GO, Debruyne FM and Schalken JA: Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. *Cancer Res* 54: 3929-3933, 1994.
- 40) Umbas R, Isaacs WB, Bringuier PP, Xue Y, Debruyne FM and Schalken JA: Relation between aberrant alpha-catenin expression and loss of E-cadherin function in prostate cancer. *Int J Cancer* 74: 374-377, 1997.
- 41) Kii I, Amizuka N, Shimomura J, Saga Y and Kudo A: Cell-cell interaction mediated by cadherin-11 directly regulates the differentiation of mesenchymal cells into the cells of the osteo-lineage and the chondro-lineage. *J Bone Miner Res* 19: 1840-1849, 2004.
- 42) Lee DM, Kiener HP, Agarwal SK, Noss EH, Watts GF, Chisaka O, Takeichi M and Brenner MB: Cadherin-11 in synovial lining formation and pathology in arthritis. *Science* 315: 1006-1010, 2007.

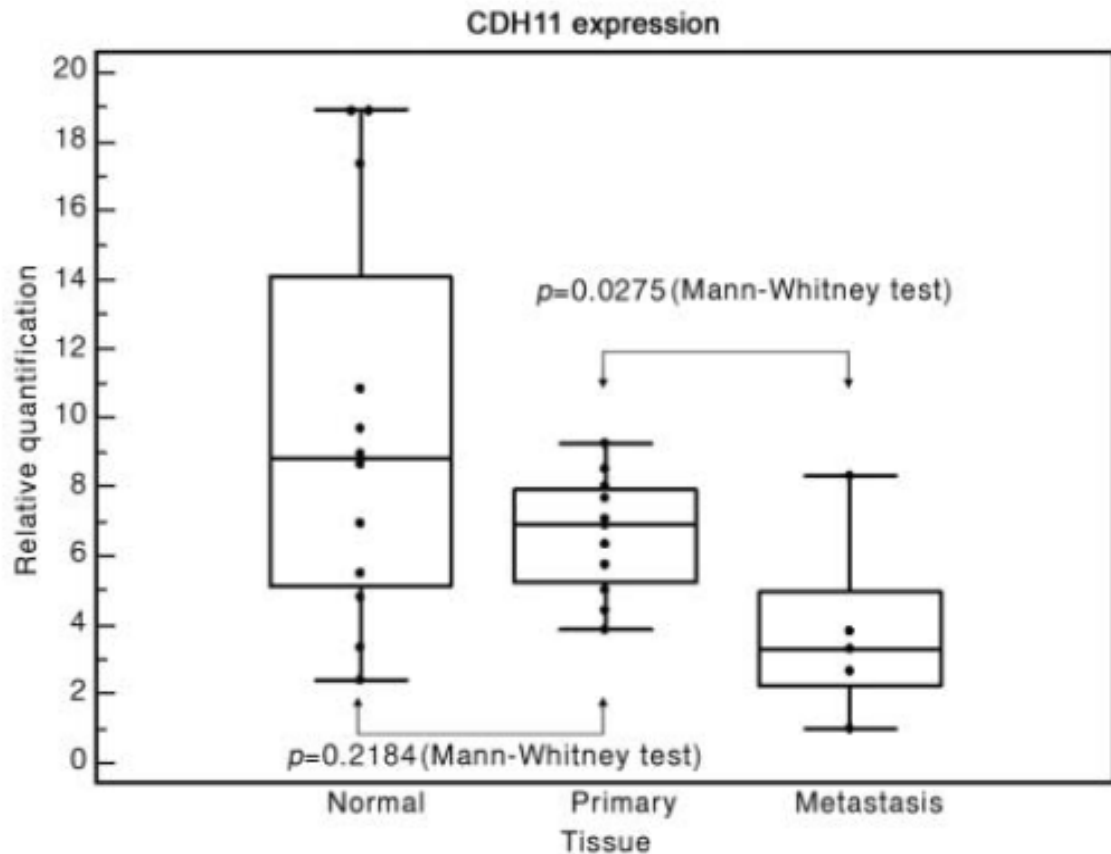


**Table 1.** Clinical data of patients from whom cell lines of primary and metastatic tumors and normal bone osteoblasts were derived.

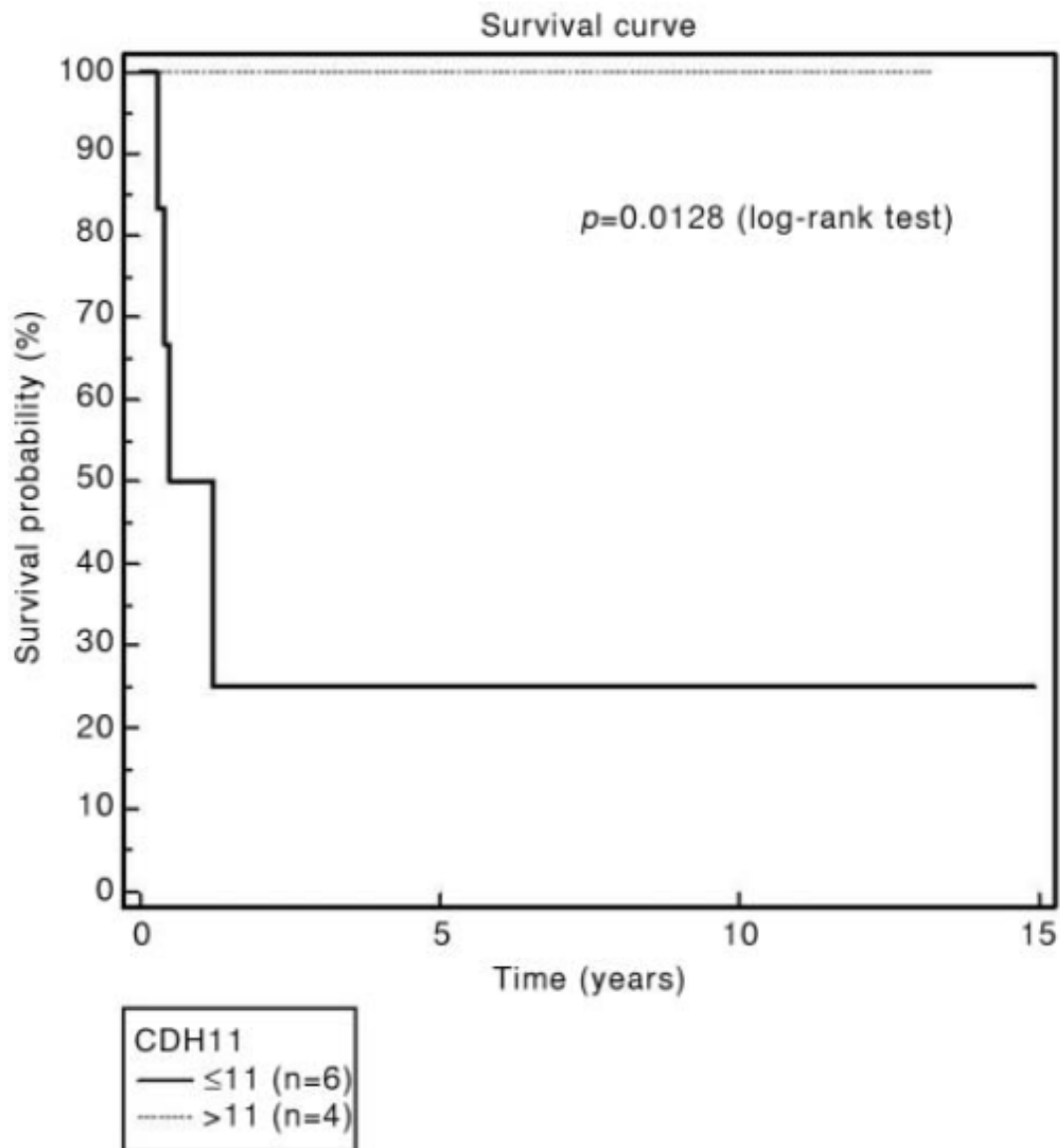
Case	Gender	Age	Site	Histological subtype	Follow-up (years)	Status
1	M	22	Fibula	Osteoblastic	6.8	alive
2	M	16	Femur	Osteoblastic	1.0	dead
3	M	15	Tibia	Fibroblastic	4.0	alive
4	F	23	Femur	Osteoblastic	4.0	alive
5	M	24	Tibia	Osteoblastic + chondroblastic	1.2	dead
6	M	16	Femur	Osteoblastic + fibroblastic	2.5	alive
7	M	11	Femur	Osteoblastic	1.4	alive
8	F	21	Femur	Osteoblastic	1.3	dead
9	F	11	Femur	Chondroblastic	2.2	alive
10	F	22	Femur	Osteoblastic + chondroblastic	1.9	alive
11	M	13	Femur	Osteoblastic	3.1	dead
12	M	16	Femur	Telangiectatic	1.4	alive
13	M	16	Tibia	Osteoblastic + chondroblastic	0.6	alive
14	F	16	Femur	Osteoblastic	0.4	alive
15	F	14	Femur	Osteoblastic	0.4	alive

**Table 2.** Clinical data of patients from whom untreated primary snap-frozen tumor samples were studied.

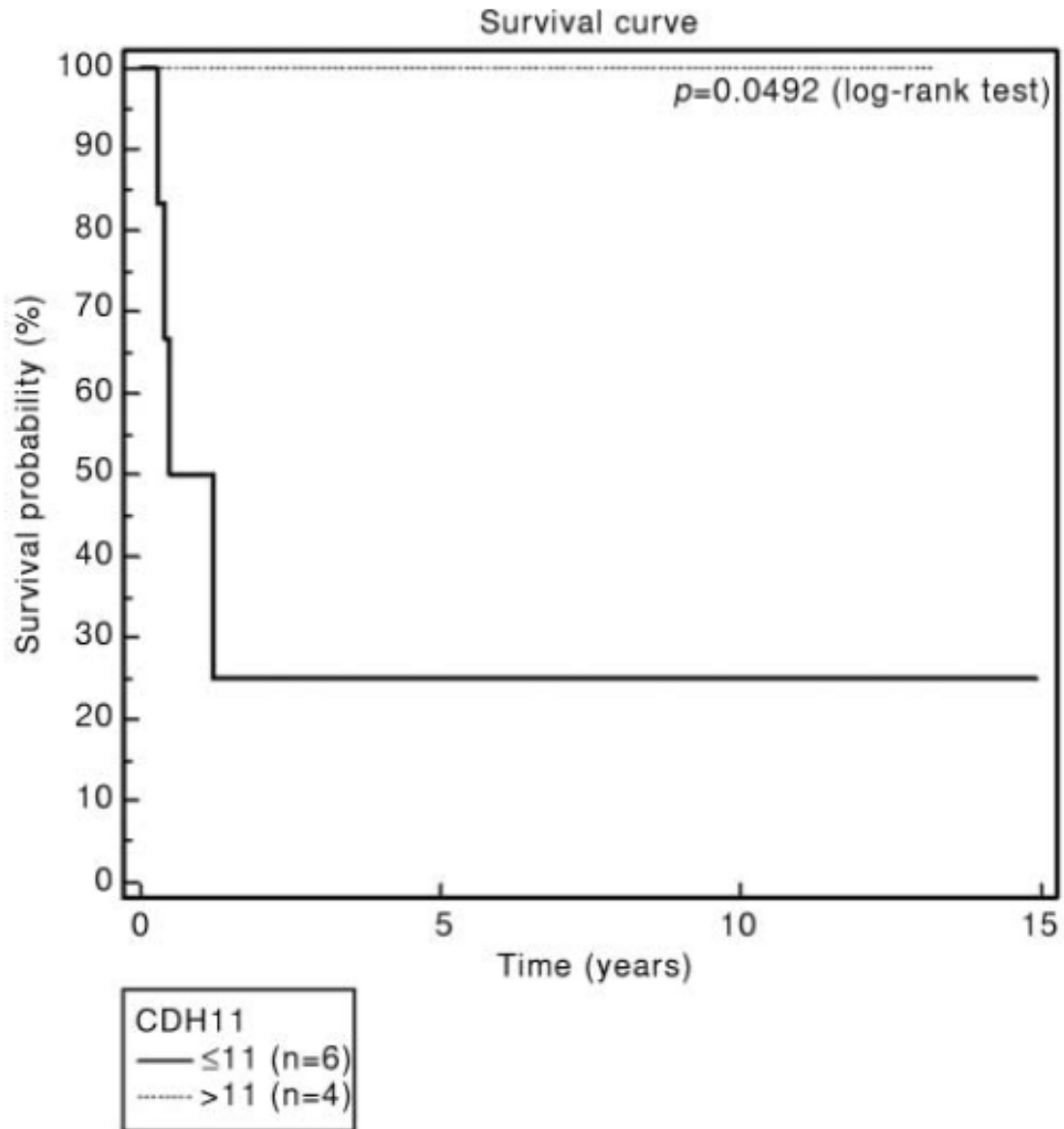
Case	Gender	Age	Site	Histological subtype	Follow-up (years)	Status
1	F	68	Femur	Osteoblastic	1.2	dead
2	F	50	Vertebra	Osteoblastic	0.3	dead
3	F	16	Tibia	Osteoblastic	14.1	alive
4	F	28	Pelvis	Osteoblastic	0.4	dead
5	F	21	Femur	Osteoblastic	13.5	alive
6	M	13	Humerus	Osteoblastic	5.7	alive
7	M	14	Tibia	Osteoblastic + fibroblastic	4.7	alive
8	M	19	Femur	N/A	4.2	alive
9	F	19	Femur	Osteoblastic + telangiectatic	0.5	dead
10	F	17	Femur	Osteoblastic	3.0	alive



**Figure 1.** CDH11 expression (mRNA level) in cell lines derived from normal osteoblasts, primary and metastatic osteosarcoma. No significant CDH11 expression difference was detected between normal and primary osteosarcoma cell lines ( $p=0.22$ ). A significant decrease ( $p=0.02$ ) was, however, observed in CDH11 expression when comparing cell lines from metastatic osteosarcoma samples and primary tumors. The levels of CDH11 were first normalized with housekeeping gene GAPDH. Relative gene expression values of CDH11 were then calculated setting the value of the lowest expression samples as one.



**Figure 2.** Overall survival curves for osteosarcoma patients from whom the cell lines were derived, related to normalized (see legend to Fig. 1) CDH11 mRNA expression levels (grouped as > or < than 5) (Kaplan-Meier survival plot). Log-rank p-value =0.01.



**Figure 3.** Overall survival curves for osteosarcoma patients from whom the biopsied samples were obtained, related to normalized CDH11 mRNA expression levels (grouped as  $>$  or  $<$  than 11) in the snap frozen samples (Kaplan-Meier survival plot). Log-rank p-value =0.04.