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Research paper

# Expression of adhesion, angiogenesis and cell cycle associated factors in osteosarcoma – A tissue microarray study

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## Abstract

**Objectives:** This study aimed to evaluate the prognostic value of selected cell adhesion, cell cycle and angiogenesis regulator, in the management of osteosarcoma, by immunohistochemistry on tissue microarray.

**Methods:** Thirty-five patients were included for the study (ages 6-74 years, median age 15 years). Tissue microarray was prepared and immunohistochemistry performed following standard protocols. Clinical outcome was measured as events (metastasis or death), chemo-response (stage), and survival by multivariate analysis.

**Results:** β-catenin staining was found only in cytoplasm and/or membranes of tumor cells in 83% of cases. Multivariate Cox regression analysis found that stage and β-catenin can predict survival. The estimated hazard ratios are 2.89 and 0.053, respectively. The 95% confidence intervals are (1.34, 6.21) and (0.006, 0.46). In other words, high stage patients have almost 3 times death risk than low stage patients. Beta-catenin positive patients have a 5% of death risk compared with beta-catenin negative patients. There was no association of marker presence

科研文章

# 骨肉瘤中粘附、 血管生成、和细胞周期相关因素的表达— 组织芯片免疫组织研究

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## 摘 要

**目的:** 本实验旨在借助组织芯片免疫织织化学染色技术，而评估细胞粘贴因子,细胞周期，以及血管生长调控等因素， 在处理骨肉溜中的临床实用价值。

**方法:** 本实验共含35例年龄在6到75岁之间(居中年龄为15岁)骨肉溜患者。组织芯片的制作和免疫组织化学染色按常规进行。临床结果以转移或死亡，对化疗的反应，以及肿瘤分期等指标而判定，预后以多变量统计学加以分析。

**结果:** β-连环蛋白(粘贴因子) 阳性染色仅见于83%病例的肿瘤细胞细胞质和/或细胞膜中。多变量的 Cox 回归分析发现肿瘤分期和β-连环蛋白可以预测生存率。预测的危险比率分别为 2.89 和至 0.053；95%的置信区间是 (1.34，6.21) 和 (0.006 , 0.46)。换言之，晚期肿瘤患者的死亡率较早期患者高三倍。与β-连环蛋白阴性病例相比较， β-连环蛋白阳性患者 有 5%的

with histological subtype or location. Presence of ezrin was detected in all cases presented with metastasis (8/8, 100%) and in about half of other cases 14/27, 51.9%, p = 0.015, Fisher's exact test).

**Conclusions:** Above data suggest a possible different activation status of β-catenin in osteosarcoma compared to other cancers. Cytoplasmic/membranous accumulation of β-catenin, was associated with longer survival time. The expression of ezrin is probably a late event in tumor development and important for metastasis.

**Key words:** Osteosarcoma, beta-catenin, ezrin, tissue microarray, prognosis markers

死亡风险。肿瘤组织标记物的表达情况和组织学分类及部位无关。ezrin 表达见于所有转移瘤患者 (8/8, 100%) 以及半数(14/27, 51.9%)以上的其他病例 (Fisher 精准检验: p=0.015) 。

**结论:** 以上资料显示: 和其他癌症相比, β-连环蛋白在骨肉瘤中可能有不同的激活状态。有细胞浆或细胞膜β-连环蛋白积聚的患者有较长的生存期。Ezrin 蛋白表达可能是出现在肿瘤晚期, 并在肿瘤转移过程中起重要作用。

关键词: 骨肉瘤、β-连环蛋白、 ezrin、 组织芯片、 预后标志物

## Introduction

Osteosarcoma (OS) is the most common, non-haemopoietic primary malignant tumor of bone. Although a relatively rare tumor, as a few hundred cases per year in the United States, it represents one of the most common malignancies in adolescents and young adults. The clinical course is usually characterized by aggressive local growth and rapid hematogenous systemic dissemination. Over the past a few decades, multidisciplinary clinical management , which involves neoadjuvant and adjuvant systematic chemotherapy and surgery, has brought significant improvement in survival, with the five-year survival rate being around 70% [1]. Pathological evaluation, including both tissue biopsy and examination of resection specimen, play important roles in the management including providing diagnosis and prognostic evaluation. At present, response to pre-operative chemotherapy, as manifested by percentage of tumor cell necrosis, is regarded as the most sensitive indicator of survival.

Osteosarcoma patients whose tumors respond poorly to chemotherapy are at a higher risk of relapse and adverse outcome. The chemotherapy-induced tumor cell necrosis can only be assessed after surgery. It is imperative to identify biomarkers at the time of diagnosis, i.e., using tissue biopsy material, to predict response to chemotherapy. Biomarkers usually refer to specific genes and their products with biochemical features that can be used to measure the progress of disease or the effects of treatment. With recent progress in the understanding of the molecular biology of tumorgenesis, genes involved in cell-cycle checkpoint, epithelial-mesenchymal transition, and angiogenesis are believed to play key roles in pathogenesis and progression of osteosarcoma [2].

## 导言

骨肉瘤 (OS) 是最常见的非造血、原发性恶性骨肿瘤。虽然它的总体发生率不高，但在美国，每年仍有数百病例报道。在青少年和青年成人中，它代表最常见的恶性肿瘤之一。骨肉瘤的临床特征，通常为快速的局灶性生长，随后由血流而播散到全身。在过去几十年，由于多临床学科综合治疗的应用，如辅助疗法，系统化疗，加外科手术等，该病的预后已有显著改善。目前，该病的五年存活率约为70%左右[1]. 组织穿刺标本和手术切除肿瘤组织的病理学检查，在诊断和预后评估的管理中发挥了非常重要的作用。目前，手术前化疗所引起的肿瘤细胞坏死的百分比是公认的、最敏感的存活指标。

对化疗反应不敏感的骨肉瘤病人，其复发的风险显著增高，预后欠佳。化疗诱发的肿瘤细胞坏死，只能在手术后方能确定。故此，利用组织活检标本，以组织芯片技术检测出相应生物标记物，在诊断时判定肿瘤对化疗的反应至关重要。生物标记物通常是指某些基因或者基因产物。这些基因或者基因产物可用来观测病情的发展或者治疗的效果。由于在对肿瘤发生生物学的分子机理的不断深入了解，目前已有充分证据表明：与细胞分裂、上皮一结缔组织转换，及血管生长相关的基因，在骨肉瘤的病理发生和发展起着非常重要的作用[2]。

A variety of biological markers have been investigated for their prognostic value with variable results. This study aimed to assess the prognostic and biological value of biological markers involved in cell adhesion, motility, invasion and the epithelial-mesenchymal transition (ezrin and beta catenin (β-cat)), angiogenesis (vascular endothelial growth factor (VEGF) and placental growth factor (PlGF)) and cell cycle checkpoint regulation (p53 family including p53, p63 and p73; p16, and PTEN) in tumor samples of osteosarcoma patients.

## Materials and Methods

**Cases:**

All cases were identified in the course of clinical care during the 8-year period, 1996-2004. In compliance with institutional protocols and with guidelines of the Health Insurance Portability and Accountability Act (HIPAA), an approval was obtained from the Institutional Review Board of University of Medicine & Dentistry of New Jersey (UMDNJ) to conduct this retrospective review. Clinical histories, radiologic studies, operative notes, pathologic analyses, and medical records were evaluated. Samples included biopsy and resection spcecimen. Clinical outcome was measured as events (metastasis or death), chemo-response (grade), and survival by multivariate analysis. Estimate

**Tissue Microarray.**

Tissue microarray was prepared using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). A minimum of three cylindrical core biopsies (0.6mm) were taken from viable tumor (biopsy and/or resected specimens) and arrayed in a receipient paraffin tissue microarray block.

**Immunohistochemistry (IHC)**

Immunohistochemical staining was performed on representative slides from each case following the manufacturer's instructions. Briefly, 4-um sections were cut and slides were heated and deparaffinized. Endogenous peroxidases were quenched with 3% hydrogen peroxide. Then the slides were pretreated with Dako target retrieval solution for 40 minutes in steam. Slides were then placed in Tris-buffered saline for at least 5 minutes and loaded to Dako autostainer (Dako North America Inc, Carpinteria, CA). Primary antibodies and dilutions used in this study are listed in **Table 1**. The secondary and tertiary antibodies were applied following kit instructions (LSAB2 kit, K0675, Dako North America Inc, Carpinteria, CA). ''DAB'' kit

许多生物标记物已应用于临床，显示不同的效果。本实验旨在将以下生物标记物用于骨肉瘤患者的肿瘤样本, 以观察它们在评估病人预后以及肿瘤生物字特性中的作用。这些生物标记物可归类为: 参与细胞粘附、 运动、 入侵和上皮-间充质细胞转换 (ezrin 和 β- 连环蛋白，血管内皮生长因子 (VEGF) 和胎盘生长因子 (PlGF),血管生成和细胞周期调控 (p53 家族包括 p53、 p63、 p73; p16，和 PTEN) 。

## 材料和方法

**病例**

所有病案收集于 1966-2004 的 8 年间。本研究获新泽西大学医学及牙科学院（UMDNJ）审查委员会批准。并完全依照相关医疗管理，健康保险部门相关规定，如 IRB 和 HIPAA,实施。供本研究的材料含临床病历、 影像学检查、 手术笔记、 病理分析及医疗记录。标本为活检和手术切除的肿瘤组织。 临床结果以转移或死亡，化疗发应程度评断；生存期以多元统计学方法进行分析。

**组织芯片**

组织芯片是以手工方法在在一购制的载板上 (Beecher Instruments, Sun Prairie, WI）作成。至少三个园柱体状组织(0.6mm)从不同活检或手术切除肿瘤标本中取得，按序排列在一蜡块中。

**免疫组化染色**

免疫组化染色是在每一病例的代表性切片中，按抗体生产单位提供的方法完成。简言之，先自每一蜡块组织中制备 4-um 厚切片，将切片加热脱腊，以 3%的过氧化氢灭活内源性过氧化酶， 然后将切片在自 Dako 购买的抗原修复液中，于蒸气中孵育四十分钟。然后在 Tris 缓冲液中浸泡至少五分钟。然后将切片置于自动免疫染色机中（Dako, Carpinteria, 美国加州）。所用抗体及其浓度见**表 1**。二抗和三抗的应用按 LSAB2 试剂盒（K0675; Dako, Carpinteria, 美国加州）中的说明。 底物显色按

was used following the kit instruction (K3466, Dako North America Inc, Carpinteria, CA). The slides were then counterstained with hematoxylin, dehydrated, and cover-slipped.

DAB 试剂盒（Dako, Carpinteria, 美国加州） 中说明。底物显色后，切片以苏木素染液染色，再脱水封片。

**Table 1. Antibodies used in this study (表 1。在本研究中使用的抗体)**

| Abs<br>抗体 | Catalog #<br>分类号 | Manufacturer<br>生产厂家 | Dilutions<br>稀释度 |
|---|---|---|---|
| p53 | M7001 | Dako North America, Inc., Carpinteria, CA | 1:150 |
| p63 | M7247 | Dako North America, Inc., Carpinteria, CA | 1:100 |
| p73 | NCL-p73 | Leica Microsystem Inc., Bannockburn, IL | 1:100 |
| p16 | K5334 | Mtm laboratories Inc., Westborough, MA | 1:50 |
| Cat* | M3539 | Dako North America, Inc., Carpinteria, CA | 1:100 |
| PTEN | NCL-PTEN | Leica Microsystem Inc., Bannockburn, IL | 1:75 |
| Ezrin | sc-20773 | Santa Cruz Biotechnology Inc., Santa Cruz, CA | 1:500 |
| VEGF | sc-13083 | Santa Cruz Biotechnology Inc., Santa Cruz, CA | 1:5 |
| PlGF | sc-1880 | Santa Cruz Biotechnology Inc., Santa Cruz, CA | 1:25 |

* Beta-catenin; β-连环蛋白

**Evaluation of IHC**

Staining was assessed based upon semi-quantitative manual scoring. Appropriate staining (membranous, cytoplasmic or nuclear) of >5% in tumor cells was considered as positive for all markers. IHC results were evaluated independently by two pathologists (W.C. and M.H.). Discrepant results were reviewed together and discussed and a consensus was made.

**Survival analysis**

Clinical outcome was measured as events (metastasis or death), chemo-response (grade), and survival by multivariate analysis. The association of IHC staining with chemoresponse and correlated between markers were calculated by Fisher's exact test.

## Results

**Patient Information**

Thirty-five (35) patients with forty-seven specimens were included for the current study (ages 6-74 years, median age - 15 years). Patient characteristics are summarized in **table 2**. Forty-seven formalin fixed paraffin embedded blocks from 35 patients with osteosarcoma were chosen for the study. There were 23 males and 12 females. Of the 35 patients, 32 patients were high grade conventional intramedullary osteosarcoma, and 3 were Paget osteosarcomas. Time of follow up ranges from minimal

**免疫组化染色评估**

免疫组化染色评估是采用半定量的人工评分系统。任何抗体有细胞膜，细胞浆，或者细胞核的适当着色，此着色见于 5%以上的肿瘤细胞，均考虑为阳性。免疫染色的评估由 2 位病理医生（W.C. and M.H）独立进行。意见不一致的评估，通过讨论而求一致。

**预后分析**

临床结果，如肿瘤转移或病人死亡，对化疗的反应程度，存活时间和比率等，以多元统计学方法进行分析。各抗体免疫组化染色的结果和化疗反应的相关性以 Fisher 精准方法检测。

## 结果

**病人临床资料**

从 35 个病人中， 共得 47 个肿瘤标本。病人年龄为 6 到 74 岁，居中年龄为 15 岁。病人临床资料总结于**表 2**。所有的 47 个组织块均用于本研究。病人中，22 为男性，12 为女性。就肿瘤组织学类型而言，32 例为高等级常见 intramedullary, 2 例为 Paget 骨肉瘤。病人

2.6 months to maximum 210 months, with the mean of follow-up time as 64months.

临床追踪时间最短为 2.6 个月。最长为 210 个月。居中追踪时间力 64 个月。

**Table 2. Clinical data (表 2。临床数据)**

| Patient and clinical characteristics | Values |
|---|---|
| **Number of patients** | 35 |
| **Median age at diagnosis (years)** | 15 (6-74) |
| Male (%) | 23 (65.7%) |
| Female (%) | 12 (34.3%) |
| **Histological diagnosis, subtype** | |
| High-grade conventional intramedullary osteosarcoma | 32 (91.4%) |
| Paget osteosarcomas | 3 (8.6%) |
| **Tumor locations** | |
| Femur | 22 (62.9%) |
| proximal tibia | 1 (2.9%) |
| Humerus | 5 (14.3%) |
| Ilium | 3 (8.6%) |
| Distal tibia | 3 (8.6%) |
| Foot | 1 (2.9%) |
| **Clinical stages** | |
| I | 3 (8.6%) |
| II | 22 (62.9%) |
| III | 4(11.4%) |
| IV | 5(14.3%) |
| Unknown | 1 (2.9%) |
| **Metastasis** | 8 (22.9%) |
| Lung | 5 |
| Vertebra | 2 |
| CNS | 1 |
| **Response to chemotherapy** | **25** (71.4%) |
| Good response | 14 (56%) |
| Poor response | 11 (44%) |

The response to preoperative chemotherapy was assessed on biopsy specimens according to standard protocol. Good responders were defined by more than 90% necrosis in tumor cells (no more than 10% viable tumors). Twenty-five patients had assessment of response to chemotherapy. Tumor locations included femur (22), proximal tibia (5), humerus (3), ilium (3), distal tibia (1), and foot (1). The clinical staging (based on AJCC) was as follows: Stage I (3), Stage II (22), Stage III (4), Stage IV (5) and Unknown (1). Five patients had distant metastases during initial diagnosis, 3 patients developed distant metastases within 3 to 15 months of diagnosis and 26 patients were free of metastases at last follow up.

对术前化疗反应的评估是在活检标本中，依据通用的标准方法进行。对化疗敏感的反应定义为：90％的肿瘤细胞坏死(存活肿瘤细胞不超过 10％)。在所有病人中，25 例对化疗有反应。肿瘤位置包括股骨 (22)、 (1) 胫骨远端 （3）、 胫骨近端 (1)、 髂骨 (3)、 肱骨 (5) 脚 (1)。 基于 AJCC 的临床分期标准: 第一期，3 例；第二期，22 例；第三期， 4 例； 第四期,5 例； 未知， 1 例。五名病人在初诊中有远处转移，3 例患者在初诊 3 到 15 个月发生远处转移。 26 例患者在最后随访没有发现远处转移。

Among the twenty five (25) patients with response data 13 (52%) patients had good response to chemotherapy (>90% necrosis) and 11 (48%) patients had poor response to chemotherapy (<90% necrosis).

在 25 例对化疗有反应的患者中，13 例 (52%) 患者对化疗反应良好 (> 90% 肿瘤细胞坏死); 11 例 (48%) 患者对化疗的反应欠佳(< 90% 肿瘤细胞坏死)。

**Immunohistochemistry of TMA**

Staining was assessed based upon semi-quantitative manual scoring. Appropriate staining (membranous, cytoplasmic or nuclear) of >5% in tumor cells was considered as positive for all markers. Beta-catenin staining was assessed separately in nucleus, cytoplasm or membrane but it was found only present at membrane or in cytoplasm (M/C, see discussion). Staining for Ezrin, PTEN, VEGF and PlGF was found in cytoplasm and/or at membrane. For all other markers (p53, p63, p73 and p16), positive staining referred to nuclear (N) staining.

**组织芯片的免疫组化染色**

免疫组化染色的评估，是基于一个半定量的方法。任何抗体有细胞膜，细胞浆，或者细胞核的适当着色，此着色见于 5%以上的肿瘤细胞，均考虑为阳性。B-连环蛋白染色仅发现在细胞质或细胞膜(请参见讨论)。Ezrin，PTEN、 VEGF 和 PlGF 染色被发现在细胞质和/或在细胞膜。所有其他标记 (p53、 p63、 p73 和 p16) 的阳性染色仅发现在细胞核。

The immunohistochemistry of markers are shown in **Figure 1.** The percentage of presence of the markers in tumor cells is summarized in **Table 3**: p53 = 20%, p63 = 34.2%, p73 = 11.4%, p16 = 57.1%, PTEN = 85.7%, ezrin = 62.8%, beta-catenin = 82.9%. VEGF= 85.7% and PlGF = 60%. Beta-catenin staining is found only in cytoplasm and/or membranes of tumor cells, no nuclear staining was detected.

各标记物免疫染色结果见**图 1**。**表 3** 汇总各标记物在肿瘤细胞阳性染色的百分比: p53 = 20%，p63 =34.2%，p73 34.2%= 11.4%，p16 = 57.1%，PTEN =85.7%，ezrin 85.7%= 62.8%，β-连环蛋白 = 82.9%。VEGF = 85.7%， PlGF = 60%。β-连环蛋白染色仅见于细胞质和/或肿瘤细胞膜，没有核染色阳性。

**Table 3. Immunohistochemistry results (表 3。免疫组化结果)**

| Marker<br>生物标记 | Overall Staining<br>总体情况 | Staining (%) in Metastasis<br>转移癌染色 | Staining in Good responders<br>反应良好病例染色 | Staining in poor responders<br>反应欠佳病例染色 | Staining vs. chemo-response (P value)<br>染色/化疗反应 |
|---|---|---|---|---|---|
| p53 | 9/35(25.7%) | 2/8 (25%) | 3/13 (23.1%) | 4/11 (36.4%) | 0.659 |
| p63 | 12/35 (34.2%) | 4/8 (50%) | 4/13 (30.8%) | 5/11 (45.5%) | 0.675 |
| p73 | 4/35 (11.4%) | 1/8 (12.5%) | 0/13 (0%) | 2/11 (18.1%) | 0.199 |
| p16 | 20/35 (57.1%) | 2/8 (25%) | 9/13 (69.2%) | 7/11 (63.6%) | 1 |
| PTEN | 30/35 (85.7%) | 8/8 (100%) | 11/13 (84.6%) | 8/11 (72.7%) | 0.63 |
| Ezrin | 22/35 (62.8%) | 8/8 (100%) | 8/13 (61.5%) | 8/11 (72.7%) | 0.615 |
| B-cat | 29/35 (82.9%) | 7/8 (87.5%) | 12/13 (92.3%) | 8/11 (72.7%) | 0.300 |
| VEGF | 30/35 (85.7%) | 8/8 (100%) | 11/13 (84.6%) | 9/11 (81.8%) | 1 |
| PlGF | 21/35 (60%) | 5/8 (62.5%) | 9/13(69.2%) | 8/11 (72.7%) | 0.82 |

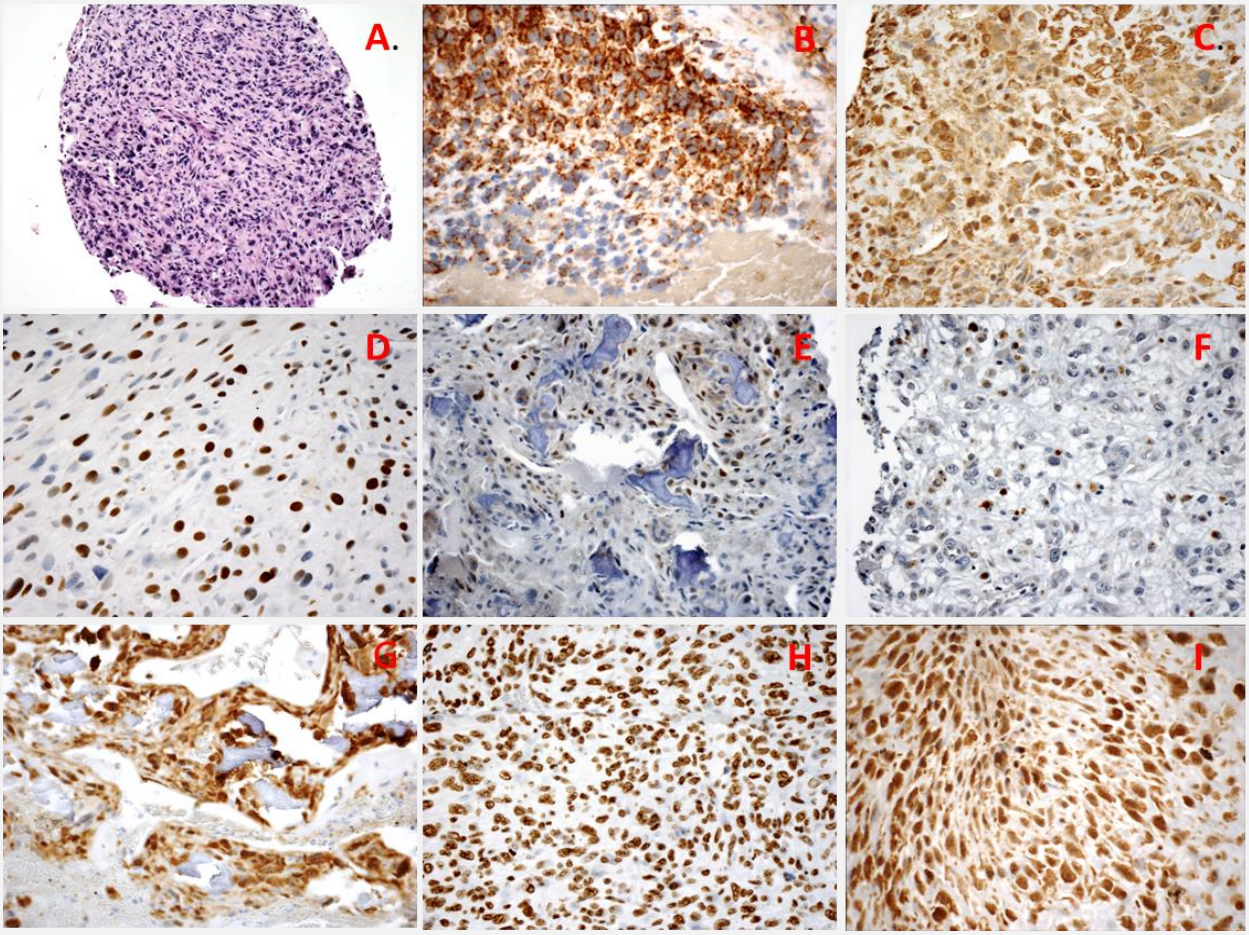


Figure 1. Representative positive immunohistochemical stains of markers in the tissue microarray. A, H&E, x100. B, β-Catenin, x600. C, Ezrin, X400. D, p53, x400. E, p63, x400. F, p73, x400. G, p16, x400. H, PTEN, x400. I. VEGF, x600.
图 1。组织芯片中的代表性免疫组化阳性染色。A、 H & E，x 100。B，β-Catenin，x 600。C，Ezrin，X 400。D，p53，x 400。E，p63，x 400。F，p73，x 400。G，p16，x 400。H，PTEN，x 400。I.VEGF，x 600。

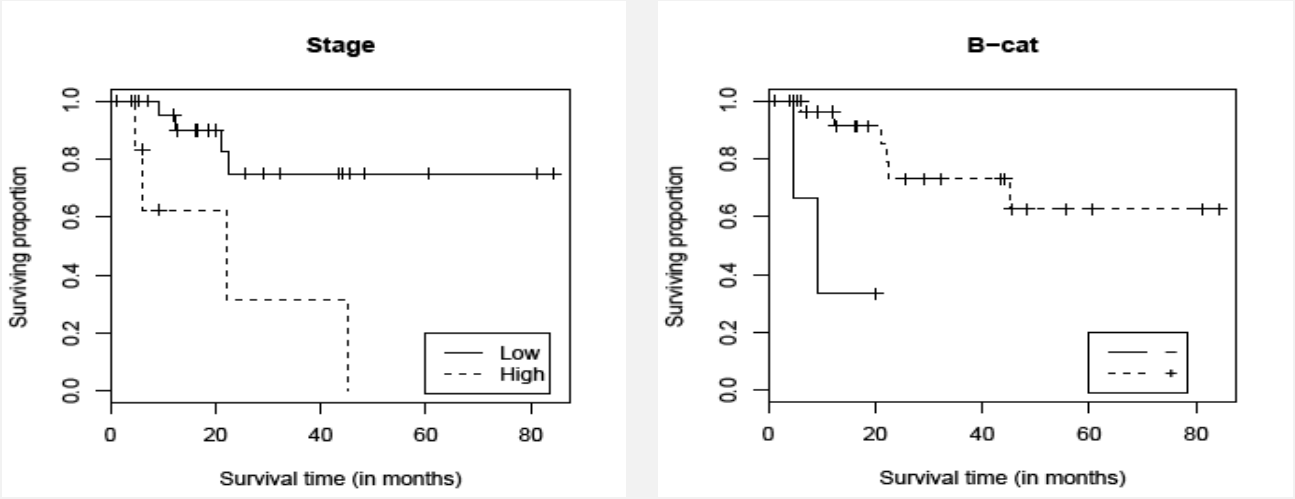


Figure 2. Survival analysis results. A, Stage (p = 0.0035). B, β-Catenin (p = 0.0013).
图 2。生存分析结果。A、 阶段 (p = 0.0035)。B、 β -Catenin (p = 0.0013)。

There is no association between histological subtype and IHC results. No consistent IHC patterns were observed within a histological subtype or location. Association of immunohistochemical staining results and response to chemotherapy was examined. The cytoplasmic/membranous staining of beta-catenin and absence of p73 nuclear staining are seen in 100% of the good responders and in approximately 70% of the poor responders (P = 0.07). Compared with pre-chemo biopsy specimen, resection specimen showed no obvious differences on staining of above markers.

IHC staining of Ezrin, VEGF and PTEN were detected in all eight cases that either presented with metastasis or developed metastasis during follow up. There is a significant difference of Ezrin staining in cases with metastasis (8/8, 100%) compared to cases without metastasis during follow-up (14/27, 51.9%, p = 0.015, Fisher's exact test). There is no significant difference between cases with metastasis and those without for VEGF or PTEN. Correlations between different markers were examined. There is a statistical significant correlation between p63 and Ezrin staining (p = 0.00090).

**Survival analysis**

Clinical variables and mark variables are examined for the association with the survival time. The log rank test is used to test individual association. The Cox regression model is employed in the multivariate analysis. Tumor stage and Beta-catenin are the significant variables to predict survival. The estimated hazard ratio for stage is 2.89 (95% confidence interval is 1.34 to 6.21). In other words, high stage patients have almost dying risk 3 times than low stage patients. Similarly, the estimated hazard ratio for Beta-catenin is 0.053 (95% confidence interval is 0.006 to 0.46). Beta-catenin positive patients have a 5% of death risk compared with Beta-catenin negative patients.

## Discussions

In the US, each year there are approximately 400 patients younger than 20 years old diagnosed as osteosarcoma each year. The management is usually a multidisciplinary approach incorporating surgery and chemotherapy. The survival is dependent on the response to pre-operative therapy, which is assessed by studying the percentage of tumor necrosis in resected specimen. Despite the progress in the management, the mortality and morbidity remains high. It would be of critical importance to find markers that can predict response to current chemotherapy

组织学亚型与免疫组化结果之间无相关性。免疫组化结果与组织学亚型或位置，没有一致模式。对免疫化学染色结果及对化疗反应研究的结果显示: β-连环蛋白的阳性细胞质和/或肿瘤细胞膜染色和缺乏 p73 核染色见于100%对化疗反应良好和大约 70%反应不良 (P = 0.07) 的病人。上面标记在手术 切除标本和化疗前活检标本的染色结果没有明显的差异。

Ezrin, VEGF 和 PTEN 的免疫组化阳性染色见于在诊断时或随后发生肿瘤转移的所有 8 例病人，。Ezrin 的免疫组化阳性染色在有肿瘤转移（8/8. 100%）和无肿瘤转移的病例中（14/27， 51.9%）有显著差异 （Fisher 精准检验 p=0.015）。VEGF 或 PTEN 的表达在有或无转移的病例无显著差异。对各生物标记物免疫染色关联性的分析揭示；P63 和 Ezrin 之间的表达有显著关联性（p = 0.00090）。

**生存分析**

临床变量和标记变量被检查与生存时间的关联。日志等级测试用于测试个体变量；Cox 回归模型用于多元变量的测试。肿瘤分期和 β-连环蛋白是预测生存率的重要变量。肿瘤分期预测的风险是 2.89 (95%的置信区间是 1.34 到 6.21)。换言之，晚期肿瘤患者死亡风险较早期患者高 3 倍。 同样，β-连环蛋白预测的风险为 0.053 (95%可信区间为 0.006-0.46)。和 β-连环蛋白阴性的患者相比，β-连环蛋白阳性患者有 5%的死亡风险。

## 讨论

在美国，每年都有大约 400 名年龄小于 20 岁的病人，被诊断为骨肉瘤。目前的临床治疗通常采用多学科的方法，结合手术和化疗。生存期一般取决于手术前对化疗的反应。目前对化疗反应的测试是在手术切除的肿瘤标本中进行。尽管在治疗方面的进展，死亡率和致残率仍然甚高。故此，在治疗前的临床材料中（如活检标本）发现可 用于预测化疗反应的生

in pre-treatment diagnostic materials, such as biopsy specimen.

Tissue microarray (TMA) is an approach that can evaluate immunohistochemical staining on large number of cases at the same time. There are a few TMA studies of osteosarcoma evaluating the prognostic value of some markers, such as the study of p16, Ezrin, EGFR and PTEN, eukaryotic initiation factor 4E (elF4E), VEGF and some other growth factors [3-9]. Current study focused on some recently emerging markers with potential prognostic values as revealed by IHC studies in other types of malignancy, mainly cancer. These makers include molecules in cell adhesion and invasion, such as beta-catenin, a member of the Wnt pathway, and ezrin; known tumor suppressor genes including the p53 family with the two relatively newer member, p63 and p73, p16 and PTEN; and angiogenesis markers including VEGF and placental growth factor (PlGF).

Beta-catenin is an important member of the Wnt pathway, which plays important roles in development, tumorgenesis and epithelial-mesenchymal transition. In unstimulated cells, beta-catenin levels are regulated by protein complex that subject beta-catenin to degradation. So cytoplasmic beta-catenin remains low in unactivated cells. Wnt signal stimulation frees catenin from the sequestration and leads to elevation of cytoplasmic beta-catenin and the ultimate nuclear translocation. Thus, the nuclear accumulation of catenin is regarded as a sign of activation. With more understanding of this pathway in tumorgenesis, immunohistochemistry of beta-catenin is gaining more attention in neoplastic pathology [10]. In bone, Wnt pathway is a positive regulator of osteoblast. While activating mutations in beta-catenin result in increased bone mass, there were no reports of beta-catenin mutations in osteosarcoma, except one N287S mutation found in 98 cases of osteosarcoma by genotyping [11]. Iwao et al reported accumulation (or elevated expression) of beta-catenin in 4 out of 6 (66.7%) osteosarcomas [12]. Haydon RC et al. [13] found cytoplasmic and/or nuclear accumulation of beta-catenin in 70% of samples. Deng et al reported expression of beta-catenin in 60% of osteosarcoma and an associated shorter survival with the expression [14]. In current study, membranous/cytoplasmic, but not nuclear, bata-catenin staining was found in approximately 80% of the osteosarcoma cases. While some previous studies found both nuclear and cytoplasmic/membranous staining of bata-catenin, our finding of cytoplasmic/membranous, instead of nuclear accumulation, of beta-catenin in osteosarcoma is consistent with some other

物标记，对降低死亡率和致残率至关重要。

组织芯片 (TMA) 可在同一时间评估大量免疫组化染色标本。先前已有几个 TMA 研究，评估了几个生物标记物在骨肉瘤预后中的价值。这些标记包括 p16, Ezrin, EGFR, PTEN, 真核生物起始因子 4E (elF4E), VEGF, 和一些其他生长因子 [3-9] 。 本研究旨在评估几个最近在其他肿瘤 (主要为癌症）中发现的，有预测预后作用的生物标记。这些分子包括细胞粘贴和侵袭(如 β-连环蛋白， Wnt 通路中的成员), ezrin, 和肿瘤抑制因子 p53 家族的两个新成员，p63，p73; 血管生长因子 VEGF 以及胎盘生长因子 PIGF。

β-连环蛋白是 Wnt 信号传导通路的重要成员，在发育、 肿瘤发生、和上皮-间充质细胞转换中起着重要的作用 。在静态细胞中，β-连环蛋白水平受蛋白质复合体调控; 此复合体使 β-连环蛋白降解。所以在未受刺激的细胞细中，胞质 β-连环蛋白表达水平很低。Wnt 信号能刺激释放多价螯合的连环蛋白，导致细胞质中连环蛋白水平升高，并运输到细胞核中。因此，细胞核中连环蛋白的积累被认为是细胞激活的标志。由于对此通路在肿瘤发生中的更多的了解，β-连环蛋白的免疫组织化学在肿瘤病理中的应用，已受到越来越多的关注[10]。在骨中, Wnt 信号传导通路是成骨细胞的正调节因子。虽然 β-连环蛋白基因突变可造成骨质增生，但除一基因定型研究发现 98 例骨肉瘤病例含 N287S 位点突变位外，目前骨肉瘤病例中尚未发现基因突变(11)。Iwao 等报道：6 例骨肉瘤病例中，4 例(66.7%)有 β-连环蛋白堆积或升高现象[12]。Haydon 等人发现 70%的骨肉瘤样本中有细胞质和细胞核性 β-连环蛋白堆积[13]。Deng 等人报道 60% 的骨肉瘤病例有 β-连环蛋白表达；有 β-连环蛋白表达的病例生存时间较短[14]。在新近研究中，大约 80%的骨肉瘤病例有阳性 β-连环蛋白在细胞 膜或者细胞质表达，但没有核表达。 虽然一些以前的研究发现 细胞核与细胞质膜 的β-连环蛋白染色[15-17]，Kashima 等 [15] 发现 β-连环蛋白只是

previous studies [15-17]. Kashima et al [15] found that catenin was expressed only focally and weakly in cytoplasm in osteosarcoma. Ng et al [16] found no detection of nuclear catenin in osteosarcoma. Cai's recent study [17] suggested that although activated Wnt pathway may be oncogeneic in many cancers, this pathway is inactivated in osteosarcoma. The result in our study supports their findings. Furthermore, survival analysis in our study found that cytoplasmic/membranous beta-catenin accumulation, possible a sign of aberrant Wnt pathway or beta-catenin inactivation, can predict survival. The estimated hazard ratios is 0.053, i.e, beta-catenin positive patients have a 5% of dying risk compared with catenin negative patients

Ezrin is a member of the ERM (ezrin, radixin, moesin) protein family. It links cytoskeleton to cell membrane. High expression of ezrin was found in osteoarcoma patients by expression profiling [18]. Study in mouse, dog and patients suggested that ezrin is necessary for metastasis [19]. Park HR et al reported ezrin staining in 14 of 32 (43.7%) high-grade osteosarcoma [20]. Kim MS et al， 51.6% [21]; Salas S et al， 62% [22]；Ferrari S et al， 80% [23], Boldrini E et al， 76% [24]. These studies also pointed to the possible association between IHC pattern (cytoplasmic vs. cytoplasmic and membranous) and prognosis [18, 23]. A meta-analysis of ezrin immunohischemical staining suggested that positive staining seems to be associated with unfavorable overall survival and recurrence [25]. A tissue array study of ezrin and related protein in 36 osteosarcoma biopsy cases demonstrated that high expression of ezrin (a combined result of percentage and intensity) associated with increased risk of metastasis [26]. Current study revealed positive ezrin staining in 62.8% of the osteosarcoma cases studied. Presence of ezrin was detected in all cases with metastasis (at presentation or during followup) (8/8, 100%) and in about half of other cases 14/27, 51.9%, p = 0.015, Fisher's exact test). Studies have shown that ezrin may play important roles in metasis by interacting with the PI3K/AKT/mTOR and MAPK pathways, or by interacting with molecules such as CD44 or hepatocyte growth factor receptor (HGFR). But most importantly, ezrin's role in metastasis may involve its interactions with E-cadherin and integrins.

Immunohistochemical study of tumor suppressor gene products in malignancy is a complicated issue. First, attention has to be paid to the antibodies used to see whether they detect the normal vs. abnormal product, such as in the case of p53, what

局灶性，低水平地存在于骨肉瘤细胞的胞浆中；Ng 等[16]没有在骨肉瘤细胞核中检测到 β-连环蛋白；Cai 的最近研究 [17] 建议虽然激活的 Wnt 信号传导通路可能在许多癌症中有促癌功能，这一通路在骨肉瘤中是灭活的。在我们的研究中， 我们仅发现细胞质/细胞膜性 β-连环蛋白。我们的研究结果支持了他们的研究结果。此外，我们的生存分析研究发现，细胞质/细胞膜 β-连环蛋白的堆积，可能是异常 Wnt 途径或 β-连环蛋白失活的迹象。此特性可以预测生存：预测的危险比率是 0.053，即，与 β-连环蛋白阴性患者相比，β-连环蛋白阳性患者有5%的死亡风险。

Ezrin 是 ERM (ezrin、radixin, 及 moesin) 蛋白家族的成员。它链接细胞骨架和细胞膜。基因表达质谱研究表明：在骨肉瘤中，其表达水平甚高[18]。 在鼠、狗、及人体实验发现：ezrin 的表达为肿瘤转移所必需[19]。Park 等报告：43.7% (14/32)高档骨肉瘤显 ezrin 阳性染色[20]；Kim 等， 51.6%[21]; Salas， 62% [22] ; Ferrari , 80% [23]; Boldrini , 76% [24]。 这些研究还指出：免疫组化染色的类型（(细胞质/细胞质和细胞膜) 与预后之间的可能关联。[18-23] 。Ezrin 免疫组化染色的荟萃分析提示: Ezrin 的阳性染色似乎与不利的总体生存率及复发相关联[25]。一近期实验以组织芯片方法研究了 Ezrin 以及相关蛋白在 36 例骨肉瘤活检标本中的表达显示：升高的 Ezrin 表达(综合阳性细胞比率以及染色强度)与升高的肿瘤转移相关[26]。本实验显示：62.8％的骨肉瘤病例中的 Ezrin 表达升高。升高的 Ezrin 表达见于所有发生肿瘤转移的病例（8/8，100％），但只见于 14/27（51.9％）的其他病例（Fisher 精准检测：p = 0.015）。既往实验已提示：Ezrin 可通过与 PI3K/AKT/mTOR 和 MAPK 通道相互作用，或者通过与 CD44 或肝细胞生长因子受体(体) 的分子相互作用，而在肿瘤转移过程起重大作用。但最重要的是，在转移中 ezrin 的作用可能涉及其与 E-钙粘蛋白和整合素的相互作用。

免疫组织化学方法研究在恶性肿瘤中的肿瘤抑制基因产物是一个复杂的问题。第一，既往的注意力仅放在用抗体染色方法去观察是否他们可 检测正常与异常的产物，如在 p53，

IHC can detect is most likely abnormal p53. Second, it is important to understand whether the presence or loss is more important, although the statistical analysis may be the same. Finally, our understanding of a specific gene is constantly evolving, the same is true for the tumor suppressor genes, and different tissues or malignancy may have different mechanism, such as the beta-catenin mentioned above and p16.

Osteosarcoma is a malignancy frequently seen in Li-Fraumeni syndrome and p53 mutations were reported to occur in 40 to 60% of osteosarcoma [27]. Previous studies of IHC detection of p53 ranged from 27 to 38% in high grade osteosarcoma [28-30]. Our study demonstrates a similar result (25.7%). A meta-analysis [31] suggested no association of p53 status to chemotherapy response. A recent meta-analysis of p53 as biomarker in osteosarcoma suggested p53 staining is decreased [32]. Our study also detected the other two members of the p53 family, p63 in 34% and p73 in 11% of osteosarcoma. Data from Park HR et al [33] suggested that low expression of p63 and p73 is relatively common in osteosarcomas and might contribute to their molecular pathogenesis. Sasaki Y et al [34] reported that ectopic expression of p73 to culture medium induced the osteoblastic differentiation of the human osteosarcoma cell line Saos-2. All these point out the important roles played by p53, p63 and p73 in pathogenesis and therapy of osteosarcoma. And the significant association between p63 and ezrin deserves more study.

The tumor suppressor gene PTEN also called MMAC1 (mutated in multiple advanced cancers), is located on chromosome 10q23.3. Decreased expression of PTEN leads to increased levels of phospho-AKT, which results in activation of antiapoptotic proteins, inactivation of members of the forkhead family of transcription factors as well as inactivation of proteins involved in cell cycle progression (p27 and p21). Both suppression of apoptosis and induction of cell cycle are processes involved in cancer development. Inactivation of PTEN can occur as mutation, or deletion, or promoter hypermethylation. Except some studies of PTEN in cell line [35] or in animal [36], there was very few study of PTEN in osteosarcoma by immunohistochemistry [37]. In their study, Freeman et al reported detection of PTEN by IHC in 19/28 (68%) of osteosarcoma cases. In current study PTEN staining was present in 30/35 (85.7%) of osteosarcoma cases with no significant correlation with histological subtypes, survival or response

免疫组织化学最有可能检测到的是异常的 p53 。第二：我们必须认识到：虽然统计分析二者可能相同，但某些标记物的存在或丢失有着更为重要的含义。最后，我们对一个特定基因的理解也在不断发展。同样如此，抑癌基因在不同组织，或不同恶性肿瘤 （如上所述的 β-连环蛋白和 p16), 可能有不同的机制。

骨肉瘤常见于 Li-Fraumeni 综合征；p53 突变出现在 40 到 60%的骨肉瘤 [27]。先前的研究显示：在高等级骨肉瘤 p53 蛋白的免疫组化检测中，p53 阳性率介乎 27% 至 38%[28-30] 。我们的研究获得相似的结果 (25.7%)。一 p53 免疫组化染色的荟萃分析[31] 提示：化疗反应和 p53 表达没有关联。一最近的荟萃分析显示：在高等级骨肉瘤中, p53 的表达是下降的 [32] 。我们的研究还检查 p53 家族的其他两个成员，发现 34%和 11%的骨肉瘤分别表达 p73 和 p63。Park 等人的研究数据提示：低表达 p63 与 p73 在骨肉瘤中较常见，可能有助于他们的分子发病机制[33]。Sasaki 等[34] 报道对培养基加 p73 能诱导人成骨肉瘤细胞株 Saos-2 成骨分化。所有这些指出： p53、 p63 和 p73 在骨肉瘤发病机制和治疗中扮演重要的角色。P63 与 ezrin 之间的重大关联值得更多的研究。

抑癌基因 PTEN，也称为 MMAC1 (在多个晚期癌症突变)，位于染色体 10q23.3。 抑癌基因 PTEN 的表达降低能导致磷酸化 AKT 表达水平升高，其可导致抗细胞胞凋亡蛋白的激活，转录因子家族成员以及参与细胞周期进展的蛋白失活。 细胞凋亡的抑制和诱导细胞周期都是参与癌症发展的重要步骤。PTEN 基因失活可起始于突变，缺失，或启动子甲基化。除了一些在细胞系 [35] 或动物[36] 的研究外，很少有人研究抑癌基因 PTEN 在骨肉瘤的免疫组化 [37]。Freeman 等在他们的研究中报道：19/28 (68%) 的骨肉瘤病例可检测到抑癌基因 PTEN 的免疫组化染色 。在我们的研究中， 抑癌基因 PTEN 免疫组化染色，可见于 30/35 (85.7%) 的骨肉瘤病例。但是 ，PTEN 的染色与

to chemotherapy.

Benassi MS et al reported that p16 staining was seen in 61.6 % and 40% of osteosarcoma [38,39], while Tsuchiya T et al reported 81% and Maitra A et al reported 84% [40,41]. The criteria for positive were different in these studies. Recently, Borys D et al reported p16 staining present in 62% of 40 osteosarcoma cases [42] and the p16 immunoexpression was associated with response to neoadjuvant chemotherapy. In our study, p16 nuclear staining was detected in approximately 60% of cases, which is very similar to Borys' result, while no significant correlation with outcome or chemoresponse was noted.

Angiogenesis plays key roles in tumor growth, progression and metastasis, thus, is a promising target for anticancer thearpy [43]. Osteosarcoma is spread by blood and markers of angiogenesis have been the targets for numerous studies. While a recent study suggesting a significantly positive correlation between VEGF immunostaining and tumor stages [44], a meta-analysis of VEGF immunohistochemistry in osteosarcoma suggested an unknown clinical significance [45]. Although IHC staining results vary in many studies, mRNA study suggested that all osteosarcoma express VEGF [46,47]. Our qPCR data detected VEGF mRNA in all cases, even in cases with negative IHC staining (Hameed, unpublished data). These results suggest that VEGF pathway may be better as a therapeutic target instead of prognostic marker. There are conflicting data on the prognostic significance of VEGF [46, 48, 49]. Furthermore, angiogenesis is regulated by a complicated network and many pathways, numerous molecules are involved, including different ligands, receptors of the VEGF family and there are more than one isoforms for many of the molecules. Different isoforms of VEGF may have different prognostic significance [46]. Selection of these isoforms rely on more investigations. Lastly, there is also a pre- or post-treatment issue [50]. Baipai J et al reported that VEGF expression in baseline (pre-treatment) did not correlate with stage or response to chemotherapy, but VEGF expression in post-treatment viable tumor cells appeared to be a negative prognostic factor [51 ].

Current study employed immunohistochemistry using TMA as a high throughput way to assess the correlations of different markers to prognosis in osteosarcoma, including some markers rarely studied in osteosarcoma, such as p63 and p73 of the p53 family, PTEN and PlGF. These markers are involved in important cellular functions and are believed to play important

组织学亚型，生存或对化疗反应无明显相关。

Benassi 等报道，p16 染色出现在 61.6% 和 40%的骨肉瘤 [38,39]，而 Tsuchiya 报道 84%, Maitra 报道 81% [40,41]。在这些研究中，p16 阳性的标准不同。最近，Borys 等报道： p16 免疫组化染色可见于 62% 的骨肉瘤病例（N= 40）；p16 免疫组化染色与化疗的反应相关联[42] 。在我们的研究中，p16 核染色见于大约 60%的病例。我们的结果非常类似于 Borys'的结果。但是，我们发现: p16 染色结果与化疗反应无显著相关。

血管生成在肿瘤生长， 发展和转移起关键作用。因此，血管生成是抗癌治疗有希望的靶点[43]。由于骨肉瘤通过血流而扩散，许多实验已对血管生成标记进行了研究。虽然一近期实验提示：VEGF 免疫结果和肿瘤分期密切相关[44],一专注骨肉瘤 VEGF 免疫组化的荟萃分析未能得出类似结论[45]。虽然免疫组化结果在许多实验中差异甚大，但所有在骨肉瘤的实验检测出 VEGF mRNA [46,47]。我们以 qPCR 方法，在所有受检的骨肉瘤标本中（包括 VEGF 组化阴性的病例），检测到 VEGF mRNA (Hameed,未发表资料)。这些结果提示：将 VEGF 传导通路作为一个治疗靶点，可能较将之用作预后标记更有意义 。关于 VEGF 在临床预后的意义，目前争议众多[46，48，49]。同时，血管生成受一个非常复杂的调控系统，众多传导通路，以及无数活性分子（如配体，受体）所支配。许多 VEGF 家族成员有多个同源体。不同同源体可能有不同的临床意义[46]. [46]。如何选择这些同源体需要更深入的研究。最后，我们需要关注一个与治疗前与治疗后相关的问题 [50]。Baipai 等报道：VEGF 表达的基线(治疗前)与肿瘤分期和对化疗的反应无关，但是 VEGF 若仍可在治疗后存活的肿瘤细胞中检出，则可提示预后不佳 [51] 。

本实验利用组织芯片免疫组化技术，评估不同生物标记物（包括很少应用于骨肉瘤研究的 p53 家族成员 P63，P73，以及 PTEN 和 PlGF）在骨肉瘤临床预后的价值。这些标记物据信参与骨细胞的重要功能，并在骨肉瘤的发

roles in pathogenesis and metastasis in osteosarcoma. Results of our study suggested that presence of cytoplasmic/membranous beta-catenin may have prognosis values and may be associated with longer survival. The presence of beta-catenin at cytoplasm and/or membrane, but not in nuclei, suggest a possible different activation or functional status of beta-catenin in osteosarcoma, compared to what is known in many other cancers.

The result of significantly different expression of ezrin in cases presented with metastasis versus cases without metastasis suggested the expression of ezrin as a relatively late event in tumor progression and an important role for metastasis. We'd recommend immunohistochemistry of beta-atenin and ezrin performed if the biopsy confirms the diagnosis of osteosarcoma.

Like the majority of IHC studies in bone and soft tissue, there are some limitations in our study. One is the sample size, many bone and soft tissue studies suffer the problem of small sample size due to the relatively rarity of the disorders. Our sample size fell into the range of TMA studies of osteosarcoma in the literature. Second, there lack a universal consistency of IHC criteria or antibodies. Criteria for positivity vary among studies. Current study employed 5% as cutoff for positivity, some studies using a stricter criterion, such as 25 or 30% as cutoff. An argument comes from the tumor heterogeneity of osteosarcoma.

Some studies used control from normal bones and/or benign bone tumors. We feel that for prognostic studies, comparison of marker expression between cases with better or worse clinical outcomes suffice. Since the incidence of osteosarcoma is relatively low compared to many other types of malignancies, a multi-institution collaborative approach, such as utilizing the specimen in the Children Oncological Group, will be helpful. For the study of relatively rare disease such as osteosarcoma, meta-analysis is a good approach. Again, an obstacle for meta-analysis is lack of standardized or consensus of immunohistochemical evaluation criteria.

More precise and quantified molecular approaches, such as expression profile between growing bone and osteosarcoma, and between pre and post-chemotherapy, can probably provide more candidates for potential IHC markers for prognosis use. However, immunohistochemistry using TMA still can provide a high throughput tool for validation. At last, an issue worth of more investigation, is that for marker studies, especially the ones

生和转移中起重要作用。我们的实验结果提示：肿瘤细胞的细胞浆或细胞膜上存在可检出的β-连环蛋白可能有非常重要的预后价值，即病人可能会有较长的生存期。我们的实验结果更进一步提示：与其他肿瘤相比，骨肉瘤细胞中蛋白的激活或功能状态可能显著不同于其他肿瘤细胞。

转移瘤和非转移瘤细胞中显著不同水平的ezrin表达提示：在肿瘤进展过程中，ezrin的表达可能发生在肿瘤后期阶段，并在肿瘤转移中起重要作用。我们建议：如果活检证实骨肉瘤之诊断，应马上作ezrin组化染色。

像大多数在骨和软组织中的免疫组化一样，我们的实验存在一些缺陷。第一是标本量。由于骨和软组织肿瘤相对少见，许多骨和软组织的实验一般标本量都较少。本实验的标本量与文献中报道的标本量类似。第二，目前国际上对免疫组化和所用抗体尚无统一认识和标准。本实验的阳性染色定为至少为5%。其他实验采用更苛刻的标准，如25%或30%。我们的标准是基于骨肉瘤的高度异质性。

有些实验用从正常骨组织或者良性骨肿瘤作为对照。我们认为：对于预后实验来说，对照标记物在临床预后良好和不佳病例中的表达可满足实验要求。由于和其他恶性肿瘤相比，骨肉瘤相对少见。故此，多单位协作(如使用儿童肿瘤所的标本)可能有助于研究。对于研究相对少见的肿瘤，如骨肉瘤来说，荟萃分析是一个非常好的方法。然而，使用该法的障碍是：对免疫组化判断标准缺乏统一的认识和标准。

更精准和定量分子学方法（如对正常生长和骨肉瘤之骨以及化疗前后之标本进行基因质谱分析），可能有助于找到更好的预后生物标记物。然而，利用组织芯片免疫组化技术照样可提供一个十分有效的评估工具。 最后，一个值得进一步研究的课题是：在一个含多个

with many markers, is the correlation between different markers and different cellular pathways.

标记的研究中，如何评估不同标记物和不同传导通路之间的关联性。

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