Cytoplasmic Accumulation of Glycogen Synthase Kinase-3β Is Associated with Aggressive Clinicopathological Features in Human Prostate Cancer

RILE LI[1], SIBEL ERDAMAR[2], HONG DAI[1], MOHAMMAD SAYEEDUDDIN[1], ANNA FROLOV[3], THOMAS M. WHEELER[1,3] and GUSTAVO E. AYALA[1,3]

[1]Department of Pathology and [3]Scott Department of Urology, Baylor College of Medicine, Houston, Texas 77030, U.S.A.; [2]Department of Pathology, Cerrahpasa Medical College, Istanbul University, Istanbul, 34300, Turkey

Abstract. *Background: Activation of glycogen synthase kinase-3 (GSK-3) is involved in the regulation of cell growth, differentiation, mobility, proliferation and survival. However, its clinicopathologic significance remains unclear in prostate cancer (PCa). Materials and Methods: A tissue microarray was produced from 640 samples. Sections were immunostained with an antibody against the non-phosphorylated form of GSK-3(GSK-3β) and were digitized. Spearman correlation test was processed for correlations between GSK-3β and biological and clinicopathological variables. The prognostic value of GSK-3β was analyzed by Cox Regression model. Results: Cytoplasmic GSK-3β was higher in PCa than in normal prostate (mean expression index 4.55 vs. 3.50, p<0.0001). Conversely, nuclear expression was higher in normal prostate than that in PCa (3.38 vs. 2.04, p<0.0001). Cytoplasmic levels of GSK-3β were correlated with clinical stage (rho=0.095, p=0.0337), lymph node metastasis (rho=0.116, p=0.0096), extracapsular extension (rho=0.092, p=0.0392), and Gleason score (rho=0.167, p=0.0002). Increased cytoplasmic GSK-3β expression was correlated with high Ki-67 labeling index (rho=0.319, p<0.0001), low apoptotic index by TUNEL (rho=–0.118, p=0.0134), high levels of androgen receptor (rho=0.292, p<0.0001) and p-Akt (rho=0.396, p<0.0001). Patients with higher cytoplasmic levels of GSK-3β had a two-fold risk of biochemical recurrence-free survival compared to those with lower levels of GSK-3β [HR 1.934 (1.020-3.667), p=0.043]. Conclusion: Cytoplasmic accumulation of GSK-3β is potentially associated with a pro-survival mechanism that promotes PCa development and progression.*

Although early diagnosis and effective clinical management has benefited numerous prostate cancer (PCa) patients, still a great many tumors progress into advanced stages, causing severe morbidity and the second highest cause of cancer death among American men. There is an urgent need to understand the molecular mechanisms of tumor development and progression, key to the discovery of novel and effective therapeutic interventions. Pca progression is a complex biological process that involves proliferation, apoptosis, signal transduction, androgen receptor signaling, cellular adhesion and angiogenesis (1). A number of signal transduction pathways including the PI3K/Akt signaling pathway have been linked to survival strategy in PCa. The PI3K/Akt signaling pathway is known to regulate cell growth, differentiation, mobility, proliferation and survival. For example, the PI3K/Akt signaling pathway has been shown to be constitutively activated in various human tumors and is responsible for increased tumor cell survival and resistance to chemo- and radiotherapy (2-4). Of note, glycogen synthase kinase 3 (GSK-3) appears to act as the central element in the PI3K/Akt survival pathway as it has been demonstrated that activation of GSK-3 induces apoptosis, while its inhibition reduces apoptosis and enhances cell survival (5). Hence the PI3K/Akt pathway components might be potential targets for the control of the growth and spread of cancer cells.

GSK-3 was discovered and named as one of the many protein kinases that phosphorylate and inactivate glycogen synthase (6). GSK-3 is a serine/threonine kinase involved in the control of many different cellular processes including development, apoptosis and metabolism (4, 7, 8). GSK-3 is a downstream target of insulin stimulation and regulates glycogen synthase, acts as a central element of the Wnt signaling pathway and is required for pattern formation

Correspondence to: Gustavo E. Ayala, MD, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, U.S.A. Tel: +1 7137983705, Fax: +1 7137982720, e-mail: gayala@bcm.tmc.edu

Key Words: GSK-3β, prostate cancer, cell signaling pathway, apoptosis, proliferation, prognosis.

during embryonic development and regulation of cell proliferation (6). GSK-3 has a high basal activity within the cell and both insulin and Wnt stimulation lead to a decrease in kinase activity. Two GSK-3 isoforms (α and β) were cloned in mammals (9). These two kinase homolog have strong sequence conservation within their catalytic domain (7). It has been suggested that GSK-3β activity can be regulated by phosphorylation as well as by intracellular redistribution for controlling its accessibility to certain substrates(5).

Significantly, GSK-3β has been implicated in the androgen receptor (AR) signaling pathway in PCa (10-12). However, the study of GSK-3β in clinical settings remains limited (13). To the Authors' knowledge, there is no report in the literature regarding the clinical and pathological significance of GSK-3β in PCa. In this regard, GSK-3β expression was examined in a large tissue microarray to look at its clinicopathlogical significance in PCa.

Materials and Methods

Clinical and pathological characteristics. A total of 640 prostate cancer patients who had undergone radical prostatectomy were included in this study. The patients had no preoperative treatment and were operated on by a single surgeon (P.T.S.) between 1983 and 1998. Radical prostatectomy specimens from these patients were processed using the whole mount slide protocol as previously described (14). Pathological analysis pertains to evaluation of staging, pathological stage, margins, capsular penetration, seminal vesicle invasion, biopsy and prostatectomy primary and secondary Gleason grades, lymph node status, tumor volume and geographic location. The clinical and pathologic data on patients who met the entry criteria were available for analysis in the Baylor College of Medicine Prostate Cancer SPORE databank. The clinical follow-up data include prostate-specific antigen (PSA) recurrence (defined as PSA>0.4 ng/ml on two consecutive measurements), clinical metastasis and death.

Patients ranged in age from 37 to 80 years, with a mean of 62 years and a median of 62.9 years. The patients were postoperatively followed-up for an average of 43.6 months. Preoperative PSA level (Pre-PSA) was available in 486 of the cases and ranged from 0.57 to 10 ng/ml (median 7.40 ng/ml; mean 10.84 ng/ml). Lymph node (LN) metastasis was found in 35 (7%) patients, and biochemical recurrence was seen in 97 patients (19.7%). Extracapular extension (ECE) was found in 44.1% of the patients. Positive surgical margins were seen in 14.2% of the patients while seminal vesicle invasion (SVI) was found in 13.6% of the patients.

Slides from all 640 radical prostatectomy specimens were reviewed and mapped. The tissue microarrays were built by a manual tissue arrayer (Beecher Instruments, Silver Spring, MD, USA). The index tumor, defined as the largest and/or highest Gleason score was identified on the slide, and areas representative of the highest Gleason score were circled. Triplicate 0.6 mm cores were obtained from the circled areas of tumor and normal prostate tissue and transferred to a recipient paraffin block. Sausage internal controls including up to 10 different types of tissues within each 0.6 mm control core were also placed with standard controls. The database was built for every block produced including the coordinates of each core and the area and case of origin.

Immunohistochemistry and assessment. Immunostaining was performed utilizing an automated immunostainer (DAKO Corporation, Carpinteria, CA, USA). Rabbit polyclonal GSK-3β antibody (Cat# 9332; Cell Signaling Technology, MA, USA), which detects total levels of endogenous GSK-3β regardless of the status of phosphorylation was used in immunhistochemical analysis. Sections were deparaffinized in xylene, rehydrated through decreasing concentrations of alcohol ending with PBS and subjected to steam heat in 10 mM citrate buffer pH 6.0 for 20 minutes in a vegetable steamer then allowed to cool off in room temperature for an additional 10 minutes. After endogenous peroxidase activity was quenched in 3% H_2O_2 solution in distilled water, sections were incubated with 1:60 dilution of GSK-3β overnight at 4°C. Sections were washed and the bound antibody was detected using Dako Envision Plus Rabbit peroxidase Kit (DAKO) with DAB as chromogen. Sections were counterstained with hematoxylin, dehydrated and mounted. Sections of formalin fixed and paraffin-embedded human breast cancer tissue were used as positive controls. Negative controls were sections immunostained as above but instead of incubation with GSK-3β, they were incubated with normal rabbit serum.

All stained slides were digitized using an automated slide scanner (Bacus Laboratories, IL, USA) to produce an image of every dot and also to record the dot coordinates on the slide. The scoring system was from 0-3+ for both intensity of stain and the percentage of positive cells (labeling frequency percentage). For intensity, the grading scale was 0 (no detectable signal), 1+ (weak signal seen only at intermediate to high power), 2+ (moderate signal seen at low to intermediate power) and 3+ (strongest signal seen at low power). For the percentage, the scale ranged was 0 (0%), 1+ (1-33%), 2+ (34-66%), and 3+ (≥67%). The total index was obtained by multiplying the scores of intensity and percentage. Nuclear and cytoplasmic GSK-3β immunostaining were evaluated and recorded seperately. In order to look at the relationship between GSK-3β and proliferation and apoptosis, GSK-3β was tested for correlations with Akt signaling pathway marker p-Akt (phosphorylated Akt), AR, proliferation marker Ki-67 and TUNEL index. AR, Ki-67 and P-Akt data were analyzed and published previously (15, 16).

Correlations between clinicopathological parameters and GSK-3β expressions (both in nuclear and cytoplasmic) were evaluated using Spearman correlation coefficient testing. For survival analysis, the end point was biochemical recurrence of the cancer defined as a serum PSA level higher than 0.4 ng/ml on two successive measurements. Time-to-recurrence was defined as the interval between the date of surgery and the date of identification of biochemical recurrence. The predictive value of GSK-3β for recurrence-free survival was evaluated using the Kaplan-Meier actuarial analysis and the log rank test. Kaplan-Meier survival curves were constructed for patients with low and high levels of GSK-3β expression. The Cox univariate and multivariate proportional hazard models were used to determine the hazard ratios. In the multivariate analysis, the model included lymph node (LN) metastasis, surgical margins, SVI, Gleason score, ECE, UICC and PrePSA levels. The hazard ratio and its 95% confidence interval were recorded for each biomarker.

Results

Tissue availability and data interpretability. After repeated cutting, small foci of cancer can be lost due to three-dimensional changes of tissue cores. The antigen retrieval
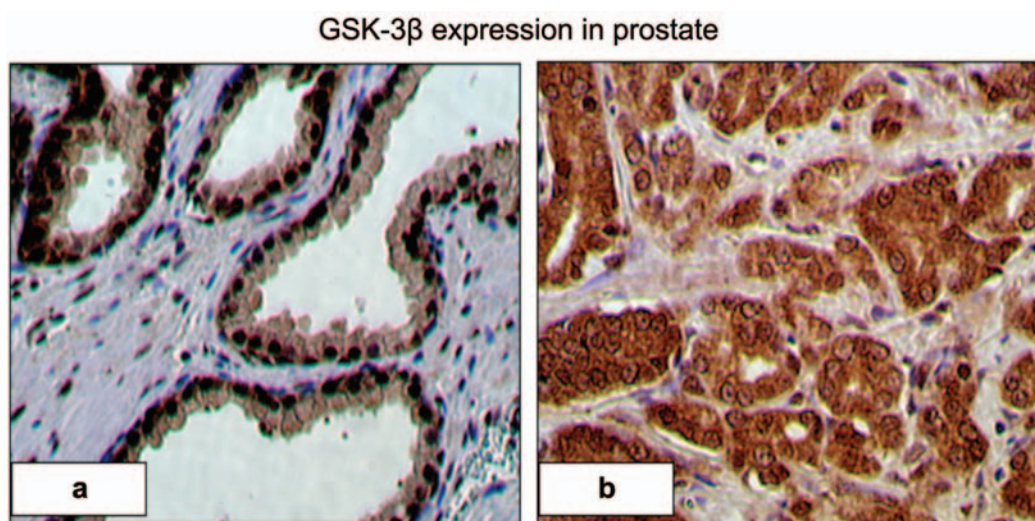
Figure 1. *GSK-3β expression in normal prostate tissue (a) and in prostate cancer tissue (b). GSK-3β was highly expressed in the nuclei (blackish brown) but almost negligible in cytoplasm of normal prostate epithelia. In contrast, GSK-3β was highly expressed in cytoplasm of cancerous epithelial cells (dark brown) while nuclear GSK-3β was lower compared to that in normal prostate tissue (immunohistochemistry ×200).*

process also contributed to tissue damage and/or core loss. These cores were thus disqualified and excluded from analysis. Therefore 491 normal prostate samples and 499 PCa cases were available for statistical analysis.

GSK-3β expression and its clinicopathological correlates. Intracellular localization of GSK-3β seems to be related to the nature of prostate tissue. GSK-3β expression was found in nucleus and cytoplasm in normal and cancerous epithelia of the prostate. The mean GSK-3β cytoplasmic expression in PCa (mean index 4.55) was significantly higher than in normal prostate (mean index 3.50) ($p<0.0001$). Conversely, nuclear expression was higher in normal prostate tissue (mean index 3.38) than in cancer (mean index 2.04) ($p<0.0001$) (Figure 1a, b). Therefore it is believed that nuclear expression of GSK-3β might be important in normal tissue while the increased cytoplasmic accumulation of GSK-3β might be essential in PCa. Indeed, increased cytoplasmic expression of GSK-3β was not only increased in PCa but also associated with many aggressive disease characteristics. Spearman correlation analysis showed that cytoplasmic expression of GSK-3β was correlated with more advanced clinical stage (rho=0.095, $p=0.0337$), positive LN status (rho=0.116, $p=0.0096$), ECE (rho=0.092, $p=0.0392$), and high Gleason score (rho=0.167, $p=0.0002$), although it was not correlated with PrePSA (rho=0.030, $p=0.0654$), patients' age (rho=0.028, $p=0.5399$), SVI (rho=0.042, $p=0.3452$) or surgical margins (rho=–0.070, $p=0.1053$). Nuclear expression of GSK-3β was neither correlated with any aforementioned prognostic variables nor was associated with biochemical recurrence.

Prognostic significance of GSK-3β in PCa. The prognostic significance of GSK-3β was tested in terms of nuclear and cytoplasmic expression by Kaplan-Meier survival analysis and the Cox regression model. No significant predictive value of nuclear GSK-3β was observed. Cytoplasmic GSK-3β, however, was found to be inversely associated with biochemical recurrence-free survival. An extensive search was performed for the cut-off value of cytoplasmic GSK-3β and it was found that the data were significant when cytoplasmic expression of GSK-3β was grouped into increased levels (expression index ≥2) and low levels (expression index <2). Patients with increased levels of cytoplasmic GSK-3β had a shorter recurrence-free survival than patients with low-level of cytoplasmic GSK-3β ($p=0.0074$). When clinical and pathological conditions were assumed identical, increased cytoplasmic levels of GSK-3β predict a two-fold risk of biochemical recurrence [multivariate analysis, $p=0.0434$, HR 1.934 (1.020-3.667)] (Figure 2).

Biological correlates and significance of GSK-3β. Close correlations were also found between cytoplasmic GSK-3β expression and levels of androgen receptor (rho=0.292, $p<0.0001$) and p-Akt (rho=0.396, $p<0.0001$). High levels of AR were previously demonstrated to be predictive of biochemical recurrence (15, 16). Increased levels of GSK-3β were correlated with a high Ki-67 labeling index (rho=0.390, $p<0.0001$, nuclear GSK-3β *vs*. Ki-67; rho=0.319, $p<0.0001$, cytoplasmic GSK-3β *vs*. Ki-67) but with low apoptotic index by TUNEL (rho=–0.250, $p<0.0001$, nuclear GSK-3β *vs*. apoptotic rate; rho=–0.118, $p=0.0134$, cytoplasmic GSK-3β *vs*. apoptotic rate). Increased
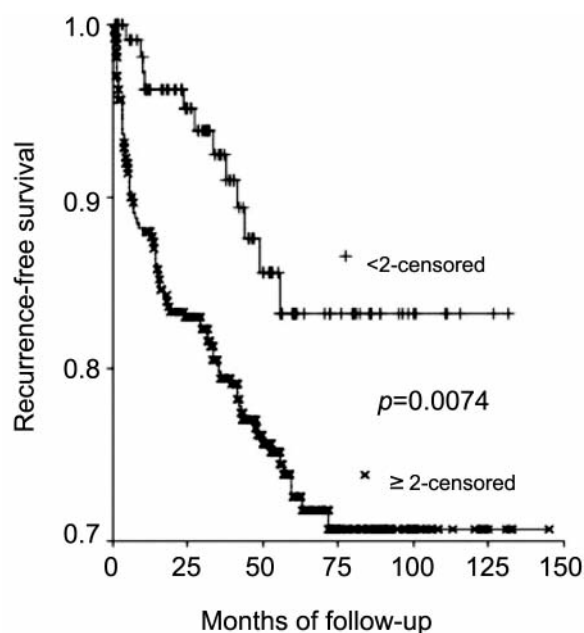
Figure 2. *Results of Kaplan-Meier survival analysis and Cox Regression analysis. GSK-3β cytoplasmic expression index in PCa. Patients with high levels (expression index ≥2) of cytoplasmic GSK-3β had a significantly shorter recurrence-free survival than patients with low-level (expression index <2) of cytoplasmic GSK-3β (p=0.0074). High level cytoplasmic GSK-3β rendered a two-fold risk of biochemical recurrence by multivariate analysis [Hazard ratio 1.934 (1.020-3.667), p=0.0434].*

cytoplasmic expression of GSK-3β appears to be associated with a pro-survival mechanism that promotes proliferation and suppresses apoptosis in PCa.

Discussion

GSK-3β is highly active in unstimulated cells and can be inactivated by mitogenic stimuli (17). In the nucleus, GSK-3β was found to co-localize with p53 following apoptotic stimuli-induced nuclear accumulation of GSK-3β (18, 19). Clearly the nucleus represents an important localization for the interactions of p53 and GSK-3β: p53 activates nuclear GSK-3β, and inhibition of GSK-3β reduces the expression of p53-regulated proteins (20). Bijur *et al.* demonstrated proapoptotic stimuli induce nuclear accumulation of GSK-3β in human neuroblastoma SH-SY5Y cells (5). Furthermore, it has been shown that the stimuli that activates apoptotic signaling cascades induce a redistribution of the GSK-3β from the cytosol to the nucleus early in the apoptotic process (5). GSK-3β activity appears to be tightly linked to its expression levels and intracellular localizations. In agreement, the presented data demonstrated GSK-3β was differentially expressed in normal prostate and PCa. It was found that nuclear GSK-3β expression was higher in normal prostate epithelia compared to PCa while cytoplasmic accumulation of GSK-3β was more common in PCa. This appears plausible since apoptotic activity prevails in normal prostate where the transfer of the GSK-3β from the cytosol to the nucleus might be facilitated by the proapoptotic regulatory mechanisms. While in cancer cells, the transfer of GSK-3β from cytosol to nucleus might be down-regulated leading to cytoplasmic accumulation of cytosolic GSK-3β. Although it is not known how the GSK-3β redistribution is regulated, cytoplasmic entrapment of GSK-3β seems to be associated with a pro-survival strategy as the data revealed that elevated cytoplasmic GSK-3β was associated with more aggressive clinicopathologic features. Increased cytoplasmic GSK-3β expression renders a two-fold risk of biochemical recurrence compared to low-level cytoplasmic GSK-3β expression in PCa. Furthermore, an increased level of cytoplasmic GSK-3β expression was correlated with increased Ki-67 index and decreased apoptotic rate. Taken together, cytoplasmic accumulation of GSK-3β appears to be associated with a pro-survival mechanism in PCa.

Currently, several regulatory pathways including AR signaling pathway, PI3K/Akt pathway, and Wnt signaling pathway have been implicated in PCa development and progression. More evidence indicates that GSK-3β acts as a central regulatory molecule in association with the interplays of Wnt signaling pathway, AR signaling pathway, and PI3K/Akt signaling cascade. In the Wnt signaling pathway, GSK-3β performs its role in the phosphorylation of β-catenin, leading to its degradation by ubiquination machinery (11, 21). GSK-3β activity is required for androgen-stimulated gene expression and suppresses AR-mediated transactivation and cell growth in PCa (10-12). Furthermore, GSK-3β activity was shown to be inhibited by increasing Ser^9 phosphorylation *via* PI3K/Akt signaling pathway(5). The presented data showed that increased GSK-3β expression (either nuclear or cytoplasmic) was closely correlated with high levels of AR and P-Akt, which were associated with aggressive features of PCa and early biochemical recurrence in PCa (15, 16). These findings suggest that GSK-3β might be intimately involved in the AR signaling pathway and PI3K-Akt signaling pathway. This provided further support that GSK-3β might be a central regulatory molecule as a coregulator of AR signaling pathway and/or as a target for PI3K/Akt pathway in PCa.

In summary, the data suggest GSK-3β cytoplasmic accumulation of GSK-3β might be associated with a pro-survival mechanism that promotes tumor development and progression in PCa. Evaluation of cytoplasmic expression of GSK-3β might be useful in prediction of disease outcome in PCa.

Acknowledgements

References

1 Quinn DI, Henshall SM and Sutherland RL: Molecular markers of prostate cancer outcome. Eur J Cancer *41*: 858-887, 2005.

2 Kolligs FT, Bommer G and Goke B: Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. Digestion *66*: 131-144, 2002.

3 Pomerantz JL and Baltimore D: Signal transduction. A cellular rescue team. Nature *406*: 26-27, 2000.

4 Ferkey DM and Kimelman D: GSK-3: new thoughts on an old enzyme. Dev Biol *225*: 471-479, 2000.

5 Bijur GN and Jope RS: Proapoptotic stimuli induce nuclear accumulation of glycogen synthase kinase-3 beta. J Biol Chem *276*: 37436-37442, 2001.

6 Harwood AJ: Regulation of GSK-3: a cellular multiprocessor. Cell *105*: 821-824, 2001.

7 Woodgett JR: Judging a protein by more than its name: GSK-3. Sci STKE 2001:RE12, 2001.

8 Kim L and Kimmel AR: GSK3, a master switch regulating cell-fate specification and tumorigenesis. Curr Opin Genet Dev *10*: 508-514, 2000.

9 Woodgett JR: Molecular cloning and expression of glycogen synthase kinase-3/factor A. Embo J *9*: 2431-2438, 1990.

10 Liao X, Thrasher JB, Holzbeierlein J *et al*: Glycogen synthase kinase-3beta activity is required for androgen-stimulated gene expression in prostate cancer. Endocrinology *145*: 2941-2949, 2004.

11 Mazor M, Kawano Y, Zhu H *et al*: Inhibition of glycogen synthase kinase-3 represses androgen receptor activity and prostate cancer cell growth. Oncogene *23*: 7882-7892, 2004.

12 Wang L, Lin HK, Hu YC *et al*: Suppression of androgen receptor-mediated transactivation and cell growth by the glycogen synthase kinase 3 beta in prostate cells. J Biol Chem *279*: 32444-32452, 2004.

13 Goto H, Kawano K, Kobayashi I *et al*: Expression of cyclin D1 and GSK-3beta and their predictive value of prognosis in squamous cell carcinomas of the tongue. Oral Oncol *38*: 549-556, 2002.

14 Wheeler TM and Lebovitz RM: Fresh tissue harvest for research from prostatectomy specimens. Prostate *25*: 274-279, 1994.

15 Ayala G, Thompson T, Yang G *et al*: High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence. Clin Cancer Res *10*: 6572-6578, 2004.

16 Li R, Wheeler T, Dai H *et al*: High level of androgen receptor is associated with aggressive clinicopathologic features and decreased biochemical recurrence-free survival in prostate: cancer patients treated with radical prostatectomy. Am J Surg Pathol *28*: 928-934, 2004.

17 Cohen P and Frame S: The renaissance of GSK3. Nat Rev Mol Cell Biol *2*: 769-776, 2001.

18 Diehl JA, Cheng M, Roussel MF and Sherr CJ: Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev *12*: 3499-3511, 1998.

19 Watcharasit P, Bijur GN, Zmijewski JW *et al*: Direct, activating interaction between glycogen synthase kinase-3beta and p53 after DNA damage. Proc Natl Acad Sci USA *99*: 7951-7955, 2002.

20 Watcharasit P, Bijur GN, Song L *et al*: Glycogen synthase kinase-3beta (GSK3beta) binds to and promotes the actions of p53. J Biol Chem *278*: 48872-48879, 2003.

21 Karim R, Tse G, Putti T *et al*: The significance of the Wnt pathway in the pathology of human cancers. Pathology *36*: 120-128, 2004.