Screening of antitumor polypeptide drug and enhanced CT imaging based on drug target analysis

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Abstract: Molecular targeted antitumor drugs is a major progress in recent years, these drugs usually target specific molecules to tumor cell signaling pathways, reduce toxicity, and can achieve individualized treatment. In this study, we screened three polypeptide proteins by yeast two hybrid systems, which could inhibit tumor growth obviously. The results of this study are expected to further develop new antitumor drugs. Moreover, by using contrast-enhanced computed tomography (CECT) imaging, this study proposes an algorithm for the computer-aided diagnosis (CAD) and classification of adrenal tumors. The experimental results demonstrate an excellent classification performance of this algorithm. Therefore, the method proposed in this paper may accurately locate and qualitatively diagnose the adrenal tumor in an effective manner, thereby providing important references for treatment.

Keywords: Antineoplastic drugs, oncology, drug target, yeast two hybrid.

INTRODUCTION

Adrenal tumor is a clinically common retroperitoneal tumor and its incidence rate exhibits a rising trend in recent years (Antonova *et al*. 2015). Adrenal gland neoplasms refer to the benign and malignant neoplasms, metastatic neoplasms and lymphomas occurring in adrenal cortex, medulla and stroma. Adrenal gland neoplasms can be classified into benign neoplasm and malignant neoplasm by nature; into non-functioning neoplasm and functioning neoplasm by endocrine function (e.g., secreting some hormone to cause high blood pressure); into cortical neoplasm, medullary neoplasm, stromal neoplasm and metastatic neoplasm by occurrence site. With the further study of the mechanism of molecular biology, researchers have turned the focus from traditional cytotoxic drugs to targeted drugs directed toward important proteins. Molecular targeted antitumor drugs is a major progress in the study of antitumor drugs in the past ten years, these drugs usually target for tumor cells to specific molecular pathways, reduce toxicity, and can achieve individualized treatment. Therefore, based on the research results of tumor biology and related disciplines, it is a major demand for medical development to find the appropriate target for drug research and development, and to select specific and high efficacy drugs. In this paper, we screened the humanized peptide aptamers library constructed by yeast two hybrid techniques with Skp2 as the target, in order to find effective anti-tumor peptide drugs.

Lack of animal model, low therapeutic target and poor drug specificity are the main bottlenecks in drug research and development. The general is a good animal model to clear the pathogenesis of disease, drug development has good predictability and complex multifactor diseases such as malignant tumor, is often difficult to find suitable targets for drug design, thus, the market specific existing anticancer drugs is relatively poor, toxic side-effects. Therefore, it is a scientific proposition to improve the targeting of antitumor drugs and highly specific without damaging normal tissues. The development of peptide drugs is an important development direction of bio pharmaceutical, compared with traditional drugs, protein drugs has its unique advantages: with the target protein with high specificity, mild toxicity, research and development costs are relatively low.

The adrenal gland neoplasms needing surgical intervention clinically are usually known as functioning neoplasms or the neoplasms highly suspected to be malignant. Adrenal gland is a vital endocrine organ in human body, and adrenal gland diseases are traditionally classified as urological diseases due to its close relationship with kidney. There are two adrenal glands in left and right sides respectively in human body, whose lower lateral margins are close to the upper medial margins of bilateral kidneys. At first blush, adrenal gland bears a strong resemblance to an adult's slightly bent little finger, and consists of head, body and tail that look like fingertip, finger prominence and finger end respectively. But a careful anatomy shows that the right adrenal gland is a triangle, while the left adrenal gland looks like a half moon. The former bestrides the innermost part of the right kidney, while the latter overhangs in the innermost part of the left kidney. They are 4.0~6.0cm long, 2.0~3.0cm wide and 0.3~0.6cm thick. A normal adrenal gland weighs roughly 4.0~5.0g. Despite the small volume of adrenal gland, the neoplasms occurring in it differ much from each other in volume. Usually, the neoplasms with a

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diameter of less than 3cm are called small neoplasm, and the minimum diameter is less than 1cm, while the maximum diameter is 10~30cm.

MATERIALS AND METHODS

Yeast two-hybrid

Screening the interaction peptide of Skp2 by yeast two hybrid technique: the yeast was transformed by PEG/Li Ac method. (1).Transformation of pGBKT7-Skp2 (208aa-390aa) into yeast Y2HGold, amplification of transformants and detection of toxicity and self activation of bait proteins; (2). The humanized peptide aptamer library 50 mu g was transformed into yeast Y2HGold-Skp2, and the peptides were screened on a low selectivity plate (SD/-Leu/-Trp/X-, -Gal/Aba+); (3).A monoclonal yeast was labeled with a highly selective plate (SD/-Leu/-Trp/-His/-Ade/X- alpha -Gal/Aba+) for further screening to reduce false-positive rates; (4).Yeast plasmids were extracted and sequenced to determine the sequence of peptide aptamers.

The bait protein toxicity and self activation detection: the conversion of GBKT7-Skp2 to Y2HGold bait vector p of yeast, with 1m L0.9% Na Cl heavy suspension, and 10 times dilution, take 100 mu L dilution were coated with SD/-Trp and SD/-Trp/X- a -Gal and SD/-Trp/X- a -Gal/Ab A tablet, 30C 3-4 d training, through the observation of yeast colonies growth and color, to determine whether the self activation Yeast two hybrid backcross experiments: Skp2 and screened peptides were transformed into Y2HGold (MATa) and Y187 (MAT alpha) in yeast colonies were selected, hybrid (200 rpm, 30 C with 20-24 h) coated low selectivity tablet (SD/-Leu/-Trp/X- a -Gal/Aba+) to determine the interaction in eukaryotic cells.

Western Blot

With the methods of Western Blot and Q PCR, the high expression of Skp2 were screened from lung cancer, breast cancer, gastric cancer and glioma tumor cells first, p21, p27, p130, TOB1, SMAD4, MYC and other substrates have a relatively low expression cell line. Plasmids expressing prokaryotic polypeptides, IPTG induced expression, SDS-PAGE staining, laid the foundation for future protein purification. Transfection of the screened eukaryotic expression plasmid corresponding Peptide or add purified protein to the corresponding tumor cells, WB cells were collected for protein detection, functional screening of these selected peptides.

CECT image

The CECT image of adrenal tumor is firstly read in, and then the LRLSM is applied to segment the tumor regions; moreover, the tumor's shape and textural features can thus be automatically extracted. Finally, SVM is adopted to classify adrenocortical tumor or medullary tumor. The neoplasm tissues in the adrenal area have complex origins, as well as various clinical and imaging features. With the development of medical imaging techniques, the accuracy of diagnosis of adrenal gland neoplasms has been significantly improved. Especially, high-resolution spiral CT and MRI can not only identify neoplasms, but also help understand the nature and blood supply of masses, and the relationship between masses and peripheral tissues. They provide a reliable imaging basis for clinical treatment.

The segmentation of adrenal tumor CECT images poses the following two problems: tumor has similar density values to those of its peripheral organs or tissues, resulting in obscure boundaries; uneven regions within the tumor may have a more distinct and clearer pseudo-boundary than that of the tumor body, for example, fatty cortical tumor and cystic-degeneration medullary tumor. To address the above problems, LRLSM may be applied to segment the tumor regions. Because LRLSM utilizes localized other than global statistical information to delineate the foreground and background, thereby better segmenting objects featuring weak boundaries and uneven regions (Damyanov *et al*. 2015; Divani *et al*. 2015; Duzagac *et al*. 2015). CECT is currently a common imaging diagnosis tool to locate the adrenal tumor. The application of CAD algorithm enables image segmentation, feature extraction and classification of adrenal tumors, thereby reducing doctors' work intensity and misdiagnosis rate. An adrenal tumor classification method based on CECT imaging is proposed in this paper. The LRLSM is firstly utilized for image segmentation, subsequently shape and textural features of tumors with significance in the clinical diagnosis are extracted in order to classify adrenocortical tumor and medullary tumor (Carvajal *et al*. 2015; Caziuc *et al*. 2015; Cetean *et al*. 2015; Cetinkunar *et al*. 2015; Chapoy-Villanueva *et al*. 2015; Chen *et al*. 2015; Cirak *et al*. 2015; Cvetanovic *et al*. 2015).

RESULTS

Screening of small molecule peptides

According to the experimental design, first through the random peptide library, yeast two hybrid system constructed by our laboratory, screening small peptide bait protein Skp2-LRR interactions, and then extract the yeast plasmid and sequenced. The correct encoding peptide sequencing, were cloned into prokaryotic expression vector and eukaryotic expression vector, prokaryotic expression system, the polypeptide transformed into Escherichia coli and expressed and purified to obtain the corresponding peptide aptamers, for tumor cell experiment; and eukaryotic expression system, through transfection methods polypeptide into tumor the cells and to verify its biological function. The yeast two hybrid low selectivity and high selective medium screening, screening to about 60 peptides, and then extract the yeast plasmid and identified by PCR, the 45 candidate peptides obtained were sequenced to remove duplicate,

not 48 bases and early termination encoding polypeptide, and finally got 11 and Skp2 the specific interaction of the peptide aptamer. In this paper, 11 peptide aptamers were synthesized and synthesized, and the peptide aptamers were synthesized by annealing. 8 small peptides were then successfully constructed into P ET28a+, as 5-1, 5-3, 6-1, 7-1, 7-2,, 8-1, 19-4 and 23-5, respectively, 23-5 sequences had an amino acid mutation, and 4-2, 12-1, 29-1 construction failed.

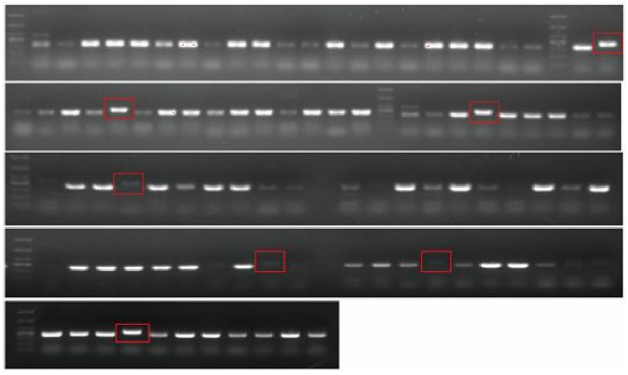

Fig.1: Results of digestion and identification

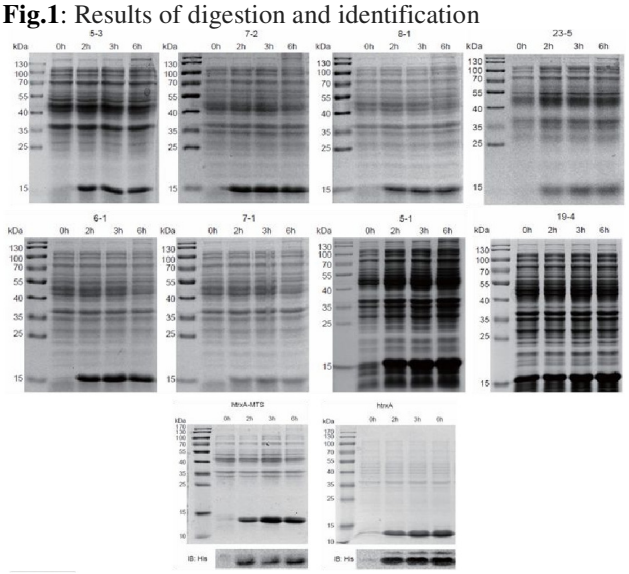

Fig.2: IPTG induced expression at different time

The correct sequencing of 8 polypeptide aptamer prokaryotic expression vector was transformed into Escherichia coli, and then pick the monoclonal plaque shake bacteria, about 12h after the addition of M IPTG1m according to the concentration of bacteria, respectively in different periods of 0h, 2h, 3H and 6h like bacteria, bacteria treatment run SDS-PAGE gel and Coomassie brilliant blue staining and destaining, results show that several polypeptide and scaffold proteins can successfully induce the expression of the amount and position correctly, as shown in fig. 2

Then the bacteria successfully induced expression of ultrasonic crushing, 8000rpm* 10min 4 DEG C by centrifugation supernatant and inclusion precipitation, with 1 x PBS inclusion precipitation cleaning lotion and 3 times, respectively for inclusion precipitation centrifugation and run SDS-PAGE glue and Coomassie brilliant blue staining, and color pictures. As shown in fig. 3. The results showed that more than 90% of the aptamers were expressed in the form of inclusion bodies. Therefore, the authors hope to promote the soluble expression of peptide aptamers by lowering the temperature.

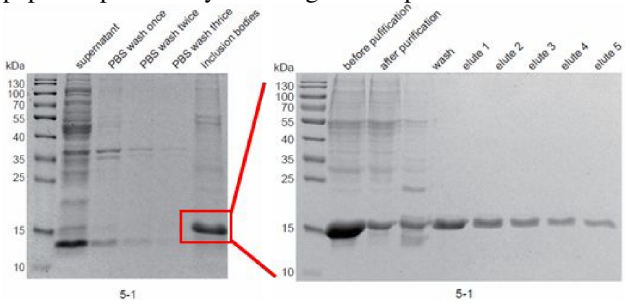

Fig. 3: NI column purification results

Screening of tumor cell lines

In the purification of small molecular peptides, hope to cell culture medium in the mode of administration, under the action of MTS peptide with peptide aptamers through the cell membrane and the effects on tumor cells, then detected the effect of peptide aptamers on tumor progression. For example, the detection of tumor cell cycle, tumor cell migration, cell proliferation and so on. So the first choice a suitable cell line, this paper respectively for lung cancer, gastric cancer, breast cancer and Skp2 and its substrates in glioma cell lines Q-PCR and Western Blot of the m RNA and protein expression level, in order to screen the expression of Skp2 is higher and the substrate relative low expression cell lines for subsequent detection.

Table 1: The results of classification performance

accuracy	95%
specificity	96%
sensibility	94%
positive prediction rate	97%
negative predication rate	90%

Detection of Skp2 and substrate of m in lung cancer cell lines RNA and protein levels showed that A549 non-small cell lung cancer compared with that of our design concept, the Skp2 has a relatively high expression, while substrate expression is relatively low, as shown in fig. 4 and 5.

CECT images

CECT images are sampled from the hospital, including 110 images related to primary aldosteronism and 191 images of pheochromocytoma. The results of all cases were verified by pathology, which shows that the adrenal tumor CAD algorithm proposed in this paper demonstrates excellent classification performance (accuracy 95%, specificity 96%, sensibility 94%, and).

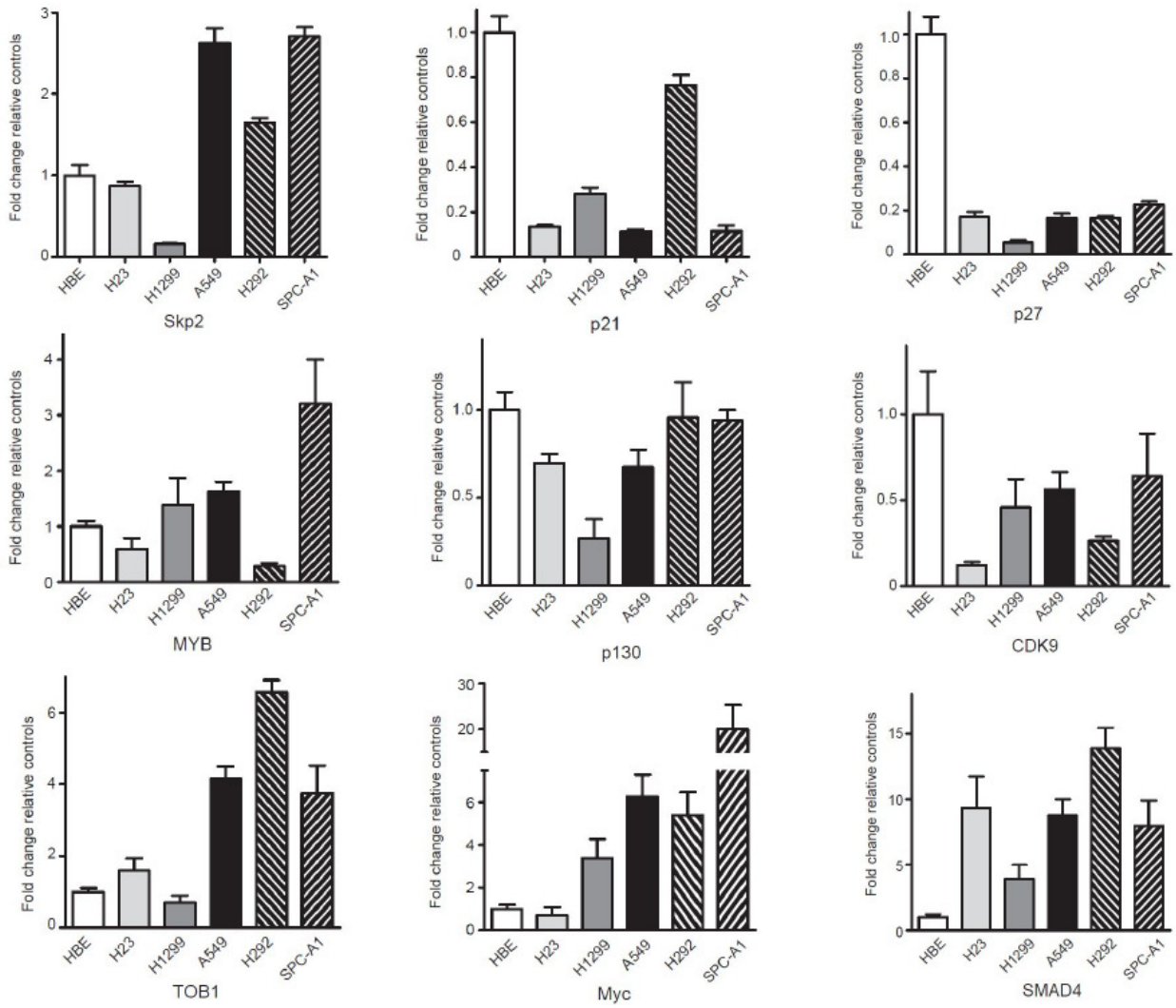

Fig. 4: Q-PCR result

Imaging has apparent advantages: first, it is minimally invasive. That is, neoplasms can be resected as long as several holes with a diameter of 1cm are opened in skin, and postoperative recovery is fast, while the traditional open surgery is subject to incisions with a diameter of over 10cm which look ugly, and postoperative recovery is slow; second, it is clear. The amplification of laparoscope makes the adrenal glands at depth close at hand, and offers a clear view that cannot be offered by the traditional open surgery. Additionally, the application of advanced incision and separation instruments also contributes to the high exactitude of surgical anatomy and little bleeding.

DISCUSSION

Skp2 (S-phase kinase associated protein-2) and Cullin, Skp1 and Rbx1 are connected to form a SCF-Skp2 complex, and then through the regulation of protease degradation pathway of the substrate (some tumor suppressor protein) degradation, thereby promoting tumor occurrence. The F-box structure of the C terminal is responsible for the specific recognition of substrate, and then ubiquitination of the substrate in the protease degradation system, and reaches 4 or more ubiquitination substrates, which can be identified and degraded by proteasome. Studies have shown that Skp2 mainly regulates the degradation of its substrate p27, thereby promoting the transformation of cells from stage G1 to stage S, thus promoting tumorigenesis. Then, studies have found that p27 is not the only target protein Skp2, inhibiting the expression of Skp2 when the cells were treated with antisense oligonucleotides, the expression of p27 did not change significantly, while the p21 has obvious changes, and the G1/S and G2/M two checkpoint regulation, suggesting that Skp2 reduced the degradation of p21 reduced cell G2/M arrest, which proves that the regulatory effect of Skp2 on p21.

Small molecular polypeptides have the characteristics of high specificity, small toxicity and low cost of research and development. In addition, researchers have used small

molecule peptides to destroy the interaction between Skp2 and Cyclin A, thereby successfully inducing selective killing of tumor cells. We use the peptide library have been constructed in our lab and tries to filter out specifically combined with Skp2 peptide aptamers and inhibit its function, so as to provide experimental basis for the development of effective anticancer drugs.

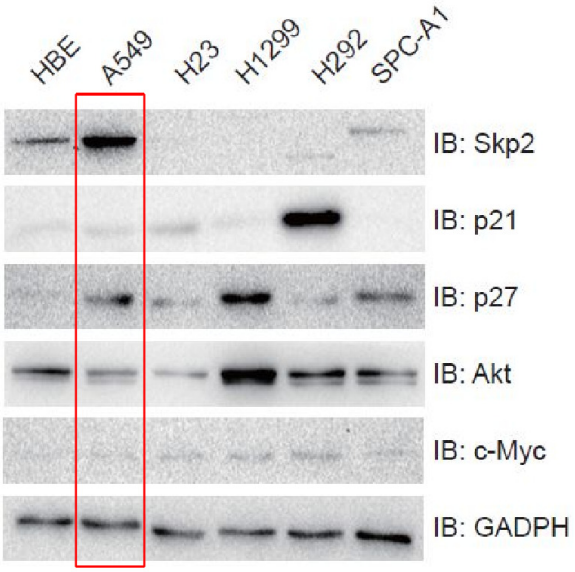

Fig. 5: Western Blot results

Also, Imaging has a significant value to the diagnosis of adrenal gland neoplasms. With the development of imaging diagnostic technologies, particularly the application of spiral CT, the localized and qualitative diagnosis ratio of adrenal gland neoplasms has been greatly improved(Abu 2017; Fang and Ruan 2017; Liu *et al*. 2017; Takahashi 2017). Spiral CT is much better than general CT in the detection of adrenal gland neoplasms, since it works continuously and can fully display adrenal gland. TLC-scanning can help detect small neoplasms effectively in the adrenal area, and for large adrenal gland neoplasms, 3D reconstruction can help distinguish between the primary sites easily and identify pseudo-tumors in the adrenal area. The adrenal gland neoplasms with a diameter<3cm are easy to localize, but it is difficult to judge the origin of large neoplasms. As reported in literature, if mass diameter is greater than 5cm, the anatomic spaces in the adrenal area will disappear, and thereby make it difficult to judge the origin of masses. Right adrenal gland neoplasms should be distinguished from lowermost liver neoplasms and uppermost kidney neoplasms, while left adrenal gland neoplasms should be distinguished from pancreatic and splenic neoplasms. Generally, liver neoplasms are not associated with high secretion. There is no intact enhanced renal cortex that can be identified after the enhancement of uppermost kidney neoplasms.

The qualitative diagnosis of adrenal gland neoplasms depends largely on clinical symptoms and biochemical examination. It can be found according to the size, density, profile, and enhancement feature of masses that different neoplasms have different CT features, and some can be diagnosed qualitatively and differentially. Hypercortisolism and primary aldosteronism are common adrenocortical adenomas, appearing as homogeneous masses, featured by high soft tissue density and preoperative localization precision. But the CT value of hypercortisolism is usually lower than soft tissue density, and approximately equal to water density due to high fattiness. 50% center of aldosteronism is liquefiable, featured by low density, looking like a cyst, but the lesion is slightly uneven. It can be differentiated from cysts under clinical and biochemical examinations. Hypercortisolism can be differentiated from neoplastic lesions since there is a lot of retroperitoneal fat in the patients with hypercortisolism. Pheochromocytoma generally has a large diameter and consists of homogeneous masses, its CT value is lower than the density of other soft tissues, there is liquefactive necrosis in its center, and 50% is multi-nodular. Enhancement scanning can reveal heterogeneous enhancement, and the primary site of large tumors can be identified through 3D reconstruction by spiral CT. Cortical adenoma: diameter<5cm generally, covered with capsule, featured by uniform density and mild enhancement. It consists mainly of functioning neoplasms, and should be differentiated from nodular hyperplasia. Hyperplastic nodule is usually multiple, with a diameter of 2.0~5.0cm, and its enhancement degree is the same as adrenal gland when being scanned. If differentiation is hard to make, chemical shift MRI examination can be performed. Cortical carcinoma: diameter>5cm generally, the masses are quasi-circular or irregularly shaped, the density is inhomogeneous, calcification is visible, the margin is fuzzy, and ring enhancement prevails. It may directly invade the adjacent tissues, most common in kidneys, inferior vena cava and regional lymph nodes, and common in liver after distant metastasis. Gangliocytoma: a neurogenic neoplasm from adrenal medulla, known as a benign neoplasm.

CONCLUSION

In this paper, the authors used Skp2 protein LRR as a bait protein, by using yeast two hybrid system of humanized peptide aptamer library was screened, and successfully screened three polypeptide protein, can effectively inhibit the degradation of the substrate of p27 or p21 Skp2 and inhibit tumor growth, indicating these peptides and proteins is expected to further development a new anticancer drug possible. At present, laparoscopic surgery is the most common method used to resect adrenal gland neoplasms.

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