Int J Clin Exp Pathol 2015;8(9):11296-11304
www.ijcep.com /ISSN:1936-2625/IJCEP0012883

Original Article

Grb7 gene amplification and protein expression by FISH and IHC in ovarian cancer

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Received July 14, 2015; Accepted August 23, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Objective: Overexpression of growth factor receptor-bound protein 7 (Grb7) has been found in numerous human cancers. The aim of this study was to evaluate the correlation between Grb7 gene amplification and protein expression in ovarian cancer (OC). Methods: We use Tissue Microarray (TMA) respectively to detect the gene amplification and protein expression of Grb7 in 90 cases OC and 10 control specimens of normal ovarian tissues by IHC and FISH. Results: The Grb7 protein expression by IHC analysis was observed in 52/90 (57.8%) OC with 3 cases (3.3%) scored 3^+ and 9 cases (10%) scored 2^+ Grb7 gene amplification by FISH analysis was successfully detectable in 6 specimens with a positive rate of 6.8% (6/88) in which immunostaining 3^+, 2^+ and negative (1^+/0) expressions of Grb7 were 100.0% (3/3), 11.1% (1/9) and 2.6% (2/76), respectively. Our data exhibited that the IHC and FISH results had a good consistency between Grb7 gene amplification and Grb7 protein expression (Kappa = 0.651, $P < 0.001$). Both the results of IHC and FISH revealed that Grb7 did not seem to have a role in OC clinicopathology. Conclusion: There is a close relationship between Grb7 gene amplification and GRB7 protein overexpression in human OC. IHC might have limited diagnostic value especially in these tumors and especially in characterizing genetically diverse borderline cases, FISH could be superior to IHC.

Keywords: Ovarian cancer, growth factor receptor-bound protein 7 (Grb7), tissue microarray, fluorescence in situ hybridization, immunohistochemistry

Introduction

Ovarian cancer (OC) remains the eighth most common cancer and the leading cause of gynecologic cancer deaths worldwide [1]. Despite many attempts to develop methods and tests to detect the disease with absent symptoms at an early stage, 70-80% of patients with OC often reach an advanced stage before the time of diagnosis [2]. As one of the most lethal gynecological malignancies found in humans, the prognosis of OC is rather unfavorable with a 5-year survival rate of only about 30% [3]. Although the use of systemic therapy in recent advances, > 75% of affected women eventually die from complications of disease progression [4]. The aggressive clinical course associated with OC underscores the need for gaining insights into the complex disease and improving therapeutic strategies. Due to remarkable heterogeneity at the clinical, cellular and molecular level, the etiology and early events in the progression of OC are poorly understood, especially highlighting the complex genetic basis. In recent reports, human OC displayed a multitude of genetic abnormalities, including deletions, amplifications and structural rearrangements exemplified by the results of The Cancer Genome Atlas (TCGA) project [5]. Gene amplification is one of the major genetic alterations in cancer, and amplicons include cancer driver genes recurrently observed in human cancers are likely to be positively selected owing to their contribution to oncogenesis. Gene amplification in cancer cells provides a means for overexpression of cancer-promoting driver genes, such as HER2 on chromosomes 17 [6]. Exploring these developments of aberration and understanding their potentially roles contributed to the pathophysiology in OC are necessary for improvements in diagnosis and specific novel-targeted therapies [7, 8].

The Grb7 (growth factor receptor-bound protein 7) is one of an Grb7 family cytosolic proteins composed of Grb7, Grb10, Grb14 and signaling proteins devoid of intrinsic enzymatic activity to affect downstream events [15]. The full-length protein is a 532-residue protein characterized by a proline-rich N-terminal domain, a Ras-associating domain, a pleckstrin homology (PH) domain, a C-terminal Src homology 2 (SH2) domain, as well as a BPS (between the pleckstrin homology and SH2) domain [16, 17]. The binding of Grb7 to its upstream partners and the target of cancer therapeutics are dictated primarily by its SH2 domain [18, 19]. It has been noted that Grb7 acted as an adapter which serves regulatory and scaffolding roles in numerous different signaling pathways [20]. Its gene is encoded in the 17q12-21 whose amplicon located < 15 kb from the HER2 gene [9]. Grb7 and HER2 were co-amplified in 15-20% of breast and gastric cancers, which may be necessary for oncogenesis, or be an acquired resistance mechanism to anti-tyrosine kinase therapy [10-14]. It has come to particular attention that the co-overexpressed of Grb7 and HER2 is covered successively in other human cancers [21-25]. Although levels of Grb7 expression in tumors are usually higher where HER2 is overexpressed, they do not always reflect HER2 levels [16, 26] with Grb7 expression under the control of complex mechanisms including the activity of the HER2 pathway itself [11]. However, Grb7 is not simply a benign side effect of HER2 overexpression. Emerging evidences have reported that Grb7 is frequently overexpressed and plays an important and independent role in the regulation of cell growth, cell migration and cell invasion of human cancers, ranging from breast [9, 16, 17, 21, 26-28], esophageal [22, 29], gastric [23, 30], lymphocytic leukemia [31], pancreatic [32], hepatocellular [33] carcinomas. Referred to OC, some studies have showed that both the mRNA and protein levels of Grb7 and its variant are frequently upregulated and play a significant role in tumorigenic functions [34, 35]. Taken together, these studies suggest that Grb7 is involved in the progression of cancers, which may serve as a potential diagnostic marker to predict drug response/resistance [36] and become a highly attractive target in the development of anticancer molecular therapeutics.

Because of the scarcely data, until now, it has been unclear what extent (over) expression of Grb7 gene reflects constitutional activation or merely reflects the physiological status of the normal progenitor cells in OC. Hence, in the current study we aimed to evaluate whether OC cells have Grb7 gene amplification, and the possible correlation between the gene by FISH and its protein expressions by IHC. In addition, we also investigated the relationship existing among Grb7 status with regard to clinicalpathology emergence in OC patients, whose correct assessment is therefore essential in guiding therapy-related decisions.

Materials and methods

Materials

The group of 100 cases of OC was derived from a commercial set of TMA slides (BC11115a; US Biomax, Inc), in which included 90 cores of tumor samples and 10 cores of normal ovarian tissues available for this study. Each of them had one core only. Approval of the protocol was obtained from the local China Ethics Committee.

Immunohistochemistry

Immunohistochemical staining for Grb7 was performed on an OC TMA (BC11115a; US Biomax, Inc.). The section was immunostained with primary polyclonal anti-Grb7 antibody (BA3733, Boster, Inc.) in 1:50 dilution. Appropriate controls were employed. Positive cells for cell membranes or cytoplasm were brown color. The percentages of positive-stained cells in tumors and normal epithelia were assessed. The proportions of positive cells were ranged from 10% to 100%, whereas the intensity of staining was scored as 0 (negative), 1^+ (weak), 2^+ (moderate), and 3^+ (strong or marked) in the most strongly stained tumor area. Immunoscoring was evaluated under an electron microscope by two independent pathologists who did not know the patients' clinical and FISH data (All were viewed at 400× magnification).

Fluorescence in situ hybridization

The FISH test was performed according to GP Medical Technologies, Ltd (Beijing, China) Grb7 DNA Probe Kit protocol. The Grb7 probe labeled red covers a 9.377-kb region of 17q12~q21 (covers the whole genome Grb7), while a control probe for CEP17 labeled green.

Table 1. The clinical pathological characteristics of patients with OC

Characteristics	N (%)
Age (years)	
< 50	44 (48.9)
≥ 50	46 (51.1)
Histopathologic type	
Serous	63 (69.3)
Mucinous	10 (11.4)
Clear cell	5 (5.7)
Endometrioid	2 (2.3)
Metastasis Others	10 (11.4)
Histological tumor grade	
Well differentiated (grade 1)	13 (14.4)
Moderate differentiated (grade 2)	19 (21.1)
Poor differentiated (grade 3)	53 (58.9)
Unknown	5 (5.6)
FIGO Stage	
I	48 (53.3)
II	15 (16.7)
III	14 (15.6)
IV	3 (3.3)
Unknown	10 (11.1)
Grb7 IHC, staining intensity score	
0	38 (42.2)
1^+	40 (44.4)
2^+	9 (10.0)
3^+	3 (3.3)
Grb7 gene amplification by FISH	
Negative	82 (93.2)
Positive	6 (6.8)
Not interpretable: 2	

Cells were counterstained with 10 μl of 4, 6-diamidino-2-phenylindole (DAPI) for 5 min and then viewed under a fluorescent microscope equipped with multiband pass filters to visualize colors simultaneously. One hundred nuclei were counted. A ratio of Grb7 signals to chromosome 17centromere signals was determined. Grb7:CEP17 ratio < 2, average Grb7 gene copy number of < 4 signals/nucleus was considered nonamplified, as well Grb7:CEP17 ratio ≥ 2.0 with CEP17 < 1.5 signals/nucleus or Grb7 < 4.0 signals/nucleus; Grb7:CEP17 ratio ≥ 2.0 or average Grb7 gene copy number ≥ 6 signals/nucleus was considered amplified; Grb7:CEP17 ratio < 2.0 and average Grb7 gene copy number 4-6 signals/nucleus, equivocal.

Statistical analysis

SPSS version 19.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis, chi-square test and the Kappa method were also used. $P < 0.05$ was defined as significant.

Results

Grb7 protein expression and correlation to other clinicopathologic parameters

The clinicopathological findings are summarized in **Table 1**. The tumors occurred in 90 women (mean age 50 years, range 22-83 years). Data describing Grb7 IHC, GRB7 FISH, OC Grade, and OC Stage were available for 100, 100, 95, and 90 patients respectively, all including ten normal ovary tissues.

Grb7 immunostaining was interpretable in all 100 cases. Immunostaining of Grb7 protein likewise 3^+, 2^+ and 1^+ (**Figure 1**) expressed in 52 (52/90; 57.8%) of 90 tumor specimens. But one case of normal ovary tissues exhibited 1^+ staining intensity. However, of the 90 tumor specimens, only 9 (9/90; 10%) were stained moderately (2^+) and 3 (3/90; 3.3%) intensively (**Table 1**). In other words, the 78 (78/90; 86.7%) remaining patients of the total tumor area showed negative ($0/1^+$) in GRB7 expressions. Nevertheless the clinicopathologic factors analysis, such as age, histopathologic type, grade and FIGO stage, showed no significant correlation with Grb7 expressions (**Table 1**) identified by chi-square test.

Grb7 gene amplification and correlation to other clinicopathologic parameters

Of 90 tumor specimens, two (2%) tumor cases were inaccessible with weak fluorescent signal. Thus, we obtained valid and easily evaluable FISH data for 88 tumor samples (**Figure 2**; **Table 1**). Based on our findings we noticed that 6 cases (6/88; 6.8%) with Grb7 amplied and nearly 82 cases (82/88; 93.2%) with negative by FISH analysis. Of six amplified cases that showed four (4/61; 6.6%) from the serous, 1 case (1/2; 50%) from the endometrioid and 1 (1/10; 10%) from metastasis. That was all cases from clear cell and mucinous carcinoma were detected without Grb7 amplification, as well as the ten cases normal ovary tissues.

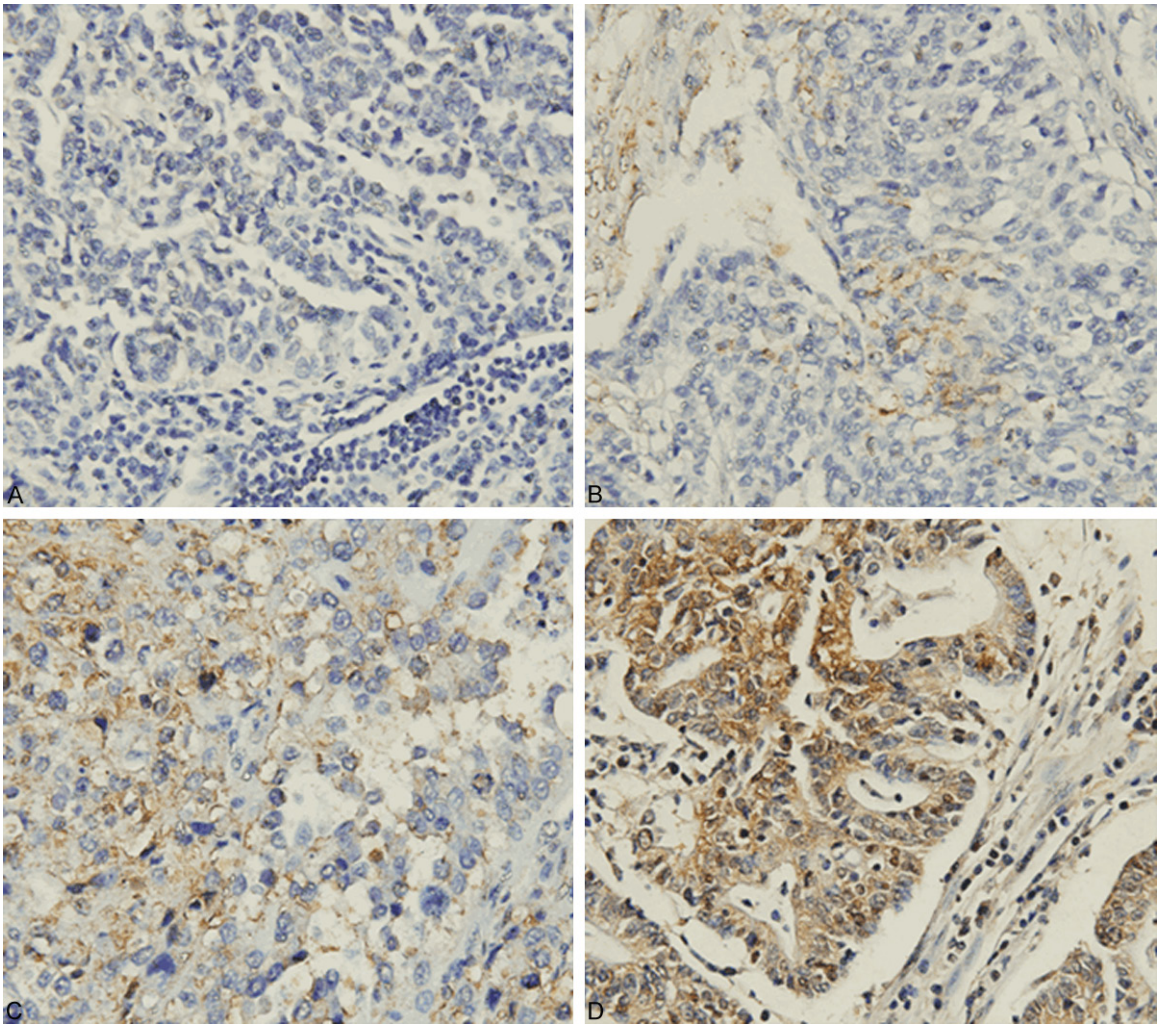

Figure 1. Representative examples of Grb7 protein expression detected by IHC (magnification, ×400). (A) Negative, (B) 1^+, (C) 2^+ and (D) 3^+ Grb7 expression. Grb7, growth factor receptor-bound protein 7; IHC, immunohistochemistry.

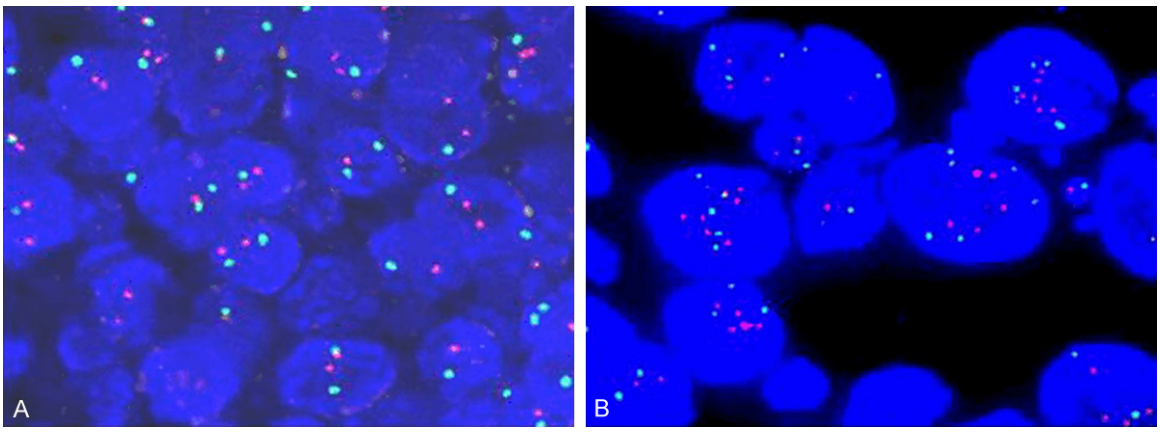

Figure 2. FISH results (A) without or (B) with Grb7 gene amplification (magnification, ×300). FISH, fluorescence in situ hybridization.

The mean age of the entire cohort was 50 years (range 22-83 years) with a mean age of 61.3 years (range 49-83, P = 0.11) among all amplified cases. However, no significant correlations

Table 2. The consistence of Grb7 gene amplification and grb7 protein expression in OC tissues

Grb7 protein (IHC)	Case/n	GRB7 gene (FISH)		Positive rate/%
		No-amplification (negative)/n	Amplification (positive)/n	
-	37	36	1	2.7
1^+	39	38	1	2.7
2^+	9	8	1	11.1
3^+	3	0	3	100.0
Total	88	82	6	6.8

were observed between amplification of Grb7 and age, FIGO stage, grade, and histopathologic type in OC (data not shown). None amplified case with FISH was observed in stage III or IV, but more common in early stage (I: 3/46; II: 2/15).

Grb7 amplification by FISH and comparison to GRB7 IHC

In order to evaluate whether our FISH evaluation score is related to and can be confirmed by Grb7 protein expression, the Grb7 status by IHC and FISH results are detailed in **Table 2**.

The 88 samples, includes all of the IHC 2^+ (9/88; 10.23%), the IHC 3^+ (3/88; 3.41%), and samples showing IHC 1^+/0 (76/88; 86.36%). Over all, IHC and FISH results were statistically significantly correlated (Kappa = 0.651, $P < 0.001$). Even if one subdivides IHC staining into "positive" (scores 2^+ and 3^+) and "negative" (scores 0 and 1^+), those categories were still excellently correlated with FISH results (Kappa = 0.389, $P < 0.001$). All the six cases amplified by FISH appeared in diversified classes of IHC, containing 3^+ (three cases), 2^+ (one case), 1^+ (one case) and 0 (one case). Most interestingly, immunoscore 3^+ was strongly correlated with amplification. Three out of six amplified cases were intensely stained resulting in IHC 3^+ cases that were all exclusively noticed in tumors with amplification. On the other hand, IHC negative cases (immunoscores 0 or 1^+) were associated with FISH negativity. The immune scores 1^+ (39/88) and 0 (37/88) each had one amplified for Grb7 validated by FISH. Tumors with Grb7 amplification presented one case with moderate (IHC 2^+) that mostly corresponded to FISH negative (8/9 cases). In addition, after excluding all 9 Grb7 moderate cases, our data showed 97.37% (n = 74/76) negative assay concordance, 100% (n = 3/3) positive assay concordance and 97.47% (n = 77/79) overall assay concordance. But had to say, our data showed 2.53% (n = 2/79) discordance with IHC negative/FISH positive.

Discussion

To our knowledge, this is the first study investigating the correlation between Grb7 gene amplification and Grb7 protein expressions in OC on Tissue Microarray by IHC and FISH techniques. Somewhat expectedly, in this study our data exhibited that the Grb7 IHC and FISH results were statistically significantly correlated and had a better consistency. Overall concordance between nonequivocal IHC and FISH results was 97.47%. All 3 cases with strong protein expression (IHC 3^+) corresponded to FISH positive, and cases with IHC 0 or 1^+ achieved a highly negative test concordance of 97.37% versus FISH results. This findings indicating that our FISH results basically have a biologic relevance where Grb7 overexpression may be due to increases in the gene copy number in chromosome 17. Most cases with morderate (IHC 2^+) staining were not amplified, although 1 of 9 cases (11.1%) was interpreted as positive by FISH whose primary reason was very close to the IHC 3^+ category in immunohistochemical interpretation. After vigilant exclusion of technical errors and evaluation biases, there were still two discordant cases interpreted carefully as IHC negative/FISH positive in TMA and verified by whole section slide assessment. The Grb7 discordant cases may be explained by limitations in the sensitivity of commercially available Grb7 antibodies or by the threshold that we required in order to score cases as "positive". Amplification of the Grb7 gene may be the primary mechanism of Grb7 protein overexpression in most cases. Besides that, alternatively, Grb7 protein expression may be also regulated by other genetic or epigenetic mechanisms, such as transcriptional, post-transcriptional, or epigenetic deregulation of the Grb7 gene. One thing we want to mention here is that we did exactly find discordant cases represent a genetically diverse group of tumors, whose biologic impacts may be of interest [37]. IHC might be limited diagnostic value in tumors and be commonly used for primary screening in current practice, but FISH could be superior to IHC,

especially in characterizing genetically diverse borderline cases. We propose to include the genomics-based information, i.e. the amplification status, obtained by FISH, in the panel of potential biomarkers.

In previous observations, Grb7 was noted in 15-20% of breast and gastric cancers studied co-amplified with HER2 [10, 11]. Inconsistent with those reports, we revealed that a lower frequency of Grb7 amplified with 6.8% based on our findings in this representative cohort of ovarian epithelial- and metastasis cell carcinomas. Thus, nearly 93.2% of these tumors do not harbor any copy number gains of the Grb7 gene. Despite all 15 clear cell and mucinous EOC (0/15; 0%) showing negative Grb7 FISH results, we found that six amplified cases respectively derived from the serous (4/61; 6.6%), the endometrioid (1/2; 50%), the metastasis (1/10; 10%). Interestingly, the surprisingly higher overall rate about 50% of Grb7 amplification in the endometrioid type need to be deep studied, even if which maybe some contingency factors attributed to a smaller number of samples. After cautious gross evaluations, however, no significant correlations were exhibited between Grb7 amplification and FIGO stage in OC, notably rather 5 cases amplified were verified as early stage (I: 3/6; II: 2/6) than none case observed in advanced stage.

For other tumors, this variation in the frequency of Grb7 expression ranged from 17-73% whose potential role of Grb7 status has been previously reported to be profound [24, 26, 27]. Regarding OC, Wang Y, David W. Chan et al, reported strong to marked staining of Grb7 and its variant, Grb7v, in 67.0% (65/97) of tumor cases on an OC tissue array (OVC1021). Besides that, they also found both the mRNA and protein levels of Grb7 are frequently upregulated in OC cell lines and clinical samples through other tests [34, 35]. Our data presented that Grb7 protein expressed in 57.8% (52/90) of tumor specimens, however, only 13.3% (12/90) were proved as Grb7 positive (IHC 2^+: 9 cases; IHC 3^+: 3 cases). And furthermore, they stated that Grb7 were frequently increased and associated with high-grade tumors and playing an important role in tumor progression [34, 35], as well as in other non-OC human malignancies [13, 26, 27, 38]. Inconsistent with prior studies we were not able to demonstrate any relationship between Grb7 overexpression and high grade of OC patients, nor clinicopathological variables, which really confused us. However, it is notable that such results should be taken with caution since the size of the groups in the Grb7 IHC 2^+ and IHC 3^+ categories were rather small (9 and 3 patients, respectively). The relationship between Grb7 and prognosis has been investigated extensively and, as noted earlier, the gene used to predict breast cancer recurrence [39]. Vinatazer, et al [24] demonstrated a correlation between higher levels of Grb7 protein expression and lower disease-free and overall survival, a finding also reported by Cobleigh et al [40] and by Nadler et al [13]. These findings correlate well with the results of the present work which taken together suggest that Grb7 holds greater clinical significance that may enter the differential diagnosis of OC. But yet, it is difficult for us to see a potential role for these tests beyond the scope of the present study. Different study populations and methodologies differences may explain this discrepancy, for instance, the use of various detecting antibodies and application of diverse scoring systems for Grb7. Furthermore, the different characteristic of samples selected in different TMA, most studies were small in size (often < 100 specimens), and thus make comparisons difficult. The present study is still not unique by including 90 patients with a very short follow-up. Hence, it is possible that analysis of much larger series might show different results.

Given to its important roles as signal transduction molecules in activating oncogenic signaling pathways, numerous studies have attempted to develop inhibitors of Grb7 in order to inhibiting aberrant activation of related signaling activities and eliminating cancer cells. For examples, targeting to the SH2 domain [19, 41-43], the non-phos-phorylated cyclic peptide, G7-18NATE for inhibiting cancer cell growth the combination of Grb7 cyclic peptide [12, 44, 45], suppressant of the specific peptide ligand [32, 44], inhibitors as Grb7-targeted molecular therapeutics [17, 44, 46], and the second virtual screen found NSC708238 among the first set of hits [47]. Grb7 inhibitors offer a new opportunity to treat cancer patients according to the genetic characteristics of their tumors and, ultimately, improve treatment outcomes. This would potentially allow for more accurate and

clinically meaningful Grb7 testing and sufficient Grb7 data for OC in the future.

Conclusion

This is the first report so far to characterize and verify GRB7 protein overexpression by IHC analysis was tight associated with the gene amplification by FISH findings in human OC. IHC may be commonly used for primary screening in current practice, but met in situ hybridization could be superior to IHC especially in characterizing genetically diverse borderline cases. The current study found that a combined approach using both IHC and FISH methodologies can optimize Grb7 testing on OC. Much more large-scale and comprehensive studies are still worth conducting that OC may represent a selected patient population for future clinical trials evaluating the utility of newly developed anti-Grb7 targeted therapeutics.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 81100443), Chongqing Yuzhong District Science and Technology Plan projects (2011-0303), Chongqing Municipal Health Bureau scientific research project (20121039). Department of Health (DOH99-FDA-43002-H), Chongqing Medical University (G098N0013) and Chongqing Medical University Hospital (CSH-2015-C-016), China, ROC.

Disclosure of conflict of interest

None.

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