Proposed Title - Bibek

血小板内在性 VEGF-A を分子標的とする新規大腸がん治療の臨床および分子生物学的研究

Clinical and Molecular Biological Research on Novel Therapy of CRC by Targeting VEGF-A Inside Platelet

Background

Conventionally viewed as major cellular components in hemostasis, coagulation and thrombosis, the association of platelets in cancer progression has remained as an emerging area of research interest in the last few years. The close interactions between cancer cells and circulating platelets play an important role in cancer growth and dissemination, and a wealth of evidence has supported a role for physiologic platelet receptors and platelet agonists in cancer progression, metastases, and angiogenesis. Platelet contains a subset of granules and these granules are rich in growth factors with mitogenic and angiogenic properties. Since, the currently marketed antiangiogeneic agents have not met the expectation that was presumed earlier, we hypothesized that targeting intra-platelet growth factors would be useful to add on the effect of anti-angiogenic agents.

During my post-doctoral fellowship, I conducted experiments and evaluated platelet functions and its kinetics in two directions: using platelet as a form a biomarker (liquid biopsy) on the prognostic and diagnostic evaluation of cancer, and understanding the fate of anti-angiogenic agents of intraplatelet growth factors. For evaluation of intra-platelet growth factor kinetics as a biomarker, we selected patients with hepatocellular carcinoma (HCC) undergoing liver resection, while for studying the impact of anti-angiogenic agent on intra-platelet growth factor, we recruited advanced colo-rectal cancer (CRC) patients who went to have received Bevacizumab therapy included in their first-line chemotherapy. The core idea of our later research was to investigate the impact of currently marketed anti-VEGF-A agents, mainly Bevacizumab, on inhibiting the intra-platelet VEGF-A and provide a novel insight in enhancing the efficacy of anti-angiogenic agents in cancer treatments.

Materials and Methods

#Intra-platelet Growth Factor as a Biomarker Experiments

Forty patients with primary HCC who went on to have partial hepatectomy were enrolled in the first study. All patients went through a thorough laboratory investigation including serum α-fetoprotein (AFP) and protein induced by vitamin K antagonist-II (PIVKA-II). Hepatic functional reserve was assessed by indocyanine green (ICG) clearance test, 99mTc-galactosyl human serum albumin (GSA) scintigraphy and Child-Pugh score. The diagnosis and staging of HCC were confirmed with triple-phase computed tomography (CT) of the abdomen.

Our Definition of Postoperative Liver Dysfunction

Postoperative liver dysfunction (LD) was assessed based on the "50-50 criteria" criteria by Balzan et al. 36. This study focused on the delayed phase of hepatic regeneration, hence, a serum bilirubin concentration >2.9 mg/dL or a PT value <50% recorded on any day within the first postoperative weeks were defined as having postoperative LD.

Follow-up

After the first-month, patients were continuously followed with abdominal echo every 3 months, and if any evidence of tumor was suspected, subsequent contrast-enhanced CT or magnetic resonance imaging (MRI) of the abdomen and non-contrast CT of the chest were performed to confirm the diagnosis.

Sample Isolation and Preparation

Venous blood was collected preoperatively (PRE OP) and after four weeks post-resection (POST OP).

Serum, plasma and fresh pure platelets were carefully isolated. The platelet pellets generated from each 2 PRP were suspended in of lysis buffer (150 mM sodium chloride, 25 mM Tris-HCl pH 7.6, 1% Tergitol-type NP-40 and 0.1 % sodium dodecyl sulfate, 1 % sodium deoxycholate in distilled water to make 100 ml solution); after incubating for 20 minutes, the lysate solution was pipetted and vortexed until the pellets were completely dissolved in the solution and intra-platelet extract was prepared.

Quantification of Cytokines

Serum, plasma and platelet extracts were analyzed together by commercially available enzyme-linked immunosorbent assay (ELISA) tests for several platelet-based growth factors and cytokines. Intra-platelet content of growth factor or cytokines were calculated and expressed per 10^6 platelets. During my post-doctoral period I focused on intra-platelet serotonin (5HT), platelet derived growth factor-BB (PDGF-BB) and von Willebrand factor (VWF).

Statistical Analysis

Statistical analyses were conducted with SPSS software (version 25.0.0; SPSS, Inc., Chicago, IL) and Graph Pad Prism (version 6.0d for Mac OS X, USA, GraphPad Software, San Diego California, USA), and were based on nonparametric tests (Mann-Whitney' s U test, Wilcoxon' s signed rank test, and Spearman' s correlation). The Fisher' s exact test was used to evaluate frequencies between categorical variables.

#For the experiments effect of anti-angiogenic agent on intra-platelet growth factor

We allocated patients who were diagnosed with advanced colorectal-cancer and not eligible for surgical resection. These patients had never received any anti-angiogenic agent before. All the patients were introduced Bevacizumab (Avastin) as a first-line combination chemotherapy agent. The patients received FOLFOX regimen, in general. And blood collection was conducted just before the introduction of Bevacizumab and before administration of the second cycle. Again, serum, plasma and pure platelet isolation was prepared. The intra-platelet extract was obtained as described earlier in the text.

Ex vivo and in vitro experiments:

The human serum, plasma and platelet extract samples were co-incubated with different doses of bevacizumab. After certain period of incubation, samples were analyzed for vascular endothelial growth factor concentration. In ex vivo model, PRP was incubated with bevacizumab and after a certain interval, the pure pellets were isolated and platelet extract was prepared for analyses, whereas in in vitro models the purely isolated fresh platelets were directly co incubated with bevacizumab. We adopted a highly standardized and upgraded protocol for preparation of these samples. Before setting the protocol, an optimum condition was established with repeating the experiments in varied conditions.

Results

Completion of Project 1: Exhausted serotonin kinetics in HCC recurrece: During my fellowship tenure, we were able to complete and publish our result on using intra-platelet as a biomarker in post-resection HCC recurrence. We observed a sharp depletion in the concentration of serum and intra-platelet 5HT after curative resection of HCC (Figure 1). In the next follow-up phase, we found that the patient with early HCC recurrence had markedly depleted serum and intra-platelet concentration (Figure 2).

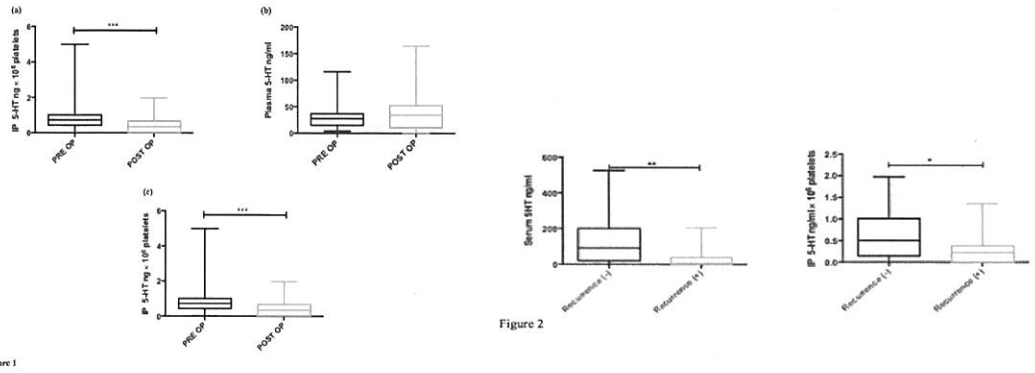


Figure 1

Figure 2

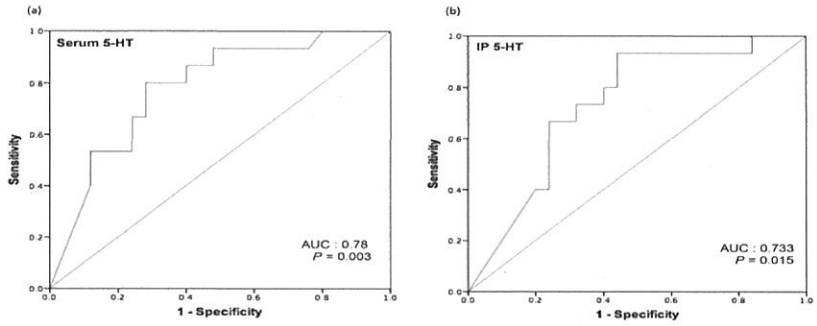


Figure 3.

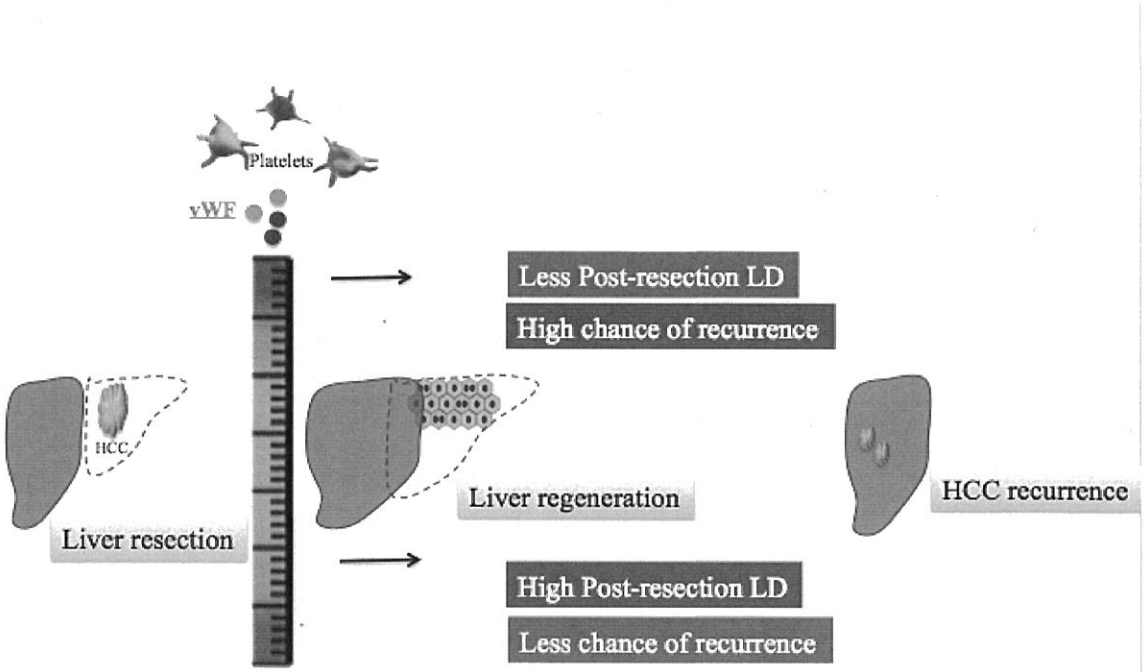
Furthermore, we examined the diagnostic potential serum and intra-platelet 5-HT by plotting receiver-operating characteristic (ROC) curves (Figure 3) for serum and intra-platelet 5-HT, which revealed significant predictive values of serum [area under curve (AUC) = 0.78; P = 0.003] and intra-platelet 5-HT (AUC = 0.733; P = 0.015). With this ROC plot, a cut-off level of 42.77 ng/ml of serum and 0.311 ng (per 10^6 platelets) of intra-platelet 5-HT was chosen to identify patients likely to develop recurrence with a specificity of 72% for serum and 68% for intra-platelet, and sensitivity of 80% or 66.7% for serum and intra-platelet respectively. This holds the positive predictive value (PPV) of 63.16% and negative predictive value (NPV) of 85.71 % for serum 5-HT, and PPV of 56 % and NPV of 77.8% for IP 5-HT. In multivariable cox regression analysis, the cut-off obtained for intra-platelet 5-HT was able to independently predict post-resection HCC recurrence, which was also statistically significant. Likewise, on the basis of these cut-off level we plotted -Meier disease-free interval (DFI) curves and found that the disease-free interval patterns differed significantly between the patients with high and low 5-HT; patients with lower serum $\{\chi\ 2\ (2) = 10.118,\ P = 0.001\}$ or Intra-platelet $\{\chi\ 2\ (2) = 4.729,\ P = 0.029\}$ 5-HT displayed a significantly shorter DFI (Figure 4). We were able to report the first evidence on intra-platelet serotonin exhaustion in patients with early cancer recurrence and this work was published in Journal of Cancer (J Cancer 2017; 8(19):3984-3991.

doi:10.7150/jca.20971). Our work got substantial attention after publication, the finding were discussed in other 2 papers published in Journal of Hepatology (IF 15.04). Moreover, a group of scientist also published a commentary based on our peculiar findings and its future relevance [doi:10.7150/jca.27497]. I am pleased to open the discussion on the unique and less described property of platelet in carcinogenesis.

Completion of Project 2: Depletion of Platelet-derived growth factor-BB in patients with HCC recurrence: After publishing our work on intra-platelet serotonin exhaustion and several queries, we were interested to investigate if the same phenomenon could occur with other platelet-related growth factor. We investigated the dynamic of platelet-derived growth factor-BB (PDGB-BB) in the same cohort of patients. PDGF-BB, in general, is regarded as a potent mitogen in various cancers. But in our investigation, we observed a significant post resection depletion of PDGF-BB in patients with early tumor recurrence. We also categorized the patient with high and low post-resection PDGF-BB and found that the cut-off value had a strong diagnostic potential in ROC curve [area under curve: 0.816, sensitivity: 80.0 %, specificity: 72.0 %, 95% CI = 0.68 0.94, P = 0.001]. This cut-off point has also a statistically significant predictive potential in cox-regression analyses and could show substantial difference in patients with or without early recurrence. This work has been submitted to Journal of Oncology and is under consideration for publication after minor revision. This manuscript is also important as a first inhuman report to reflect an inverse role of PDGF-BB in carcinogenesis.

Completion of Project 3: Bivalent properties of intra-platelet von Willebrand factor in liver regeneration and HCC recurrence: There are both structural and functional differences in properties of plasma and platelet von Willebrand factor (vWF). Role of platelet-derived is less discussed entity. There is existing evidence on the remarkable roles of plasma vWF in liver regeneration, which is a fresh discovery in the field of liver regeneration. In our study, we conducted investigation to explore the properties of intra-platelet vWF in liver regeneration. We found a significant elevation of intra-platelet vWF during liver regeneration; moreover, patients who could not achieve marked rise in post-resection intra-platelet vWF concentration were found to have increased incidence of post-resection liver dysfunction. In contrast, the patients who expressed higher intra-platelet vWF tended to have early HCC recurrence that was also statistically significant. A little is known on the role of vWF in cancer progression, however, till date no study has reported the link between intra-platelet vWF and carcinogenesis. We have accomplished this pilot study and able to introduce intra-platelet vWF and its bivalent properties in liver regeneration and cancer recurrence. Our study has highlighted

a narrow therapeutic window for implicating intra-platelet vWF in liver regeneration (Fig. 1).



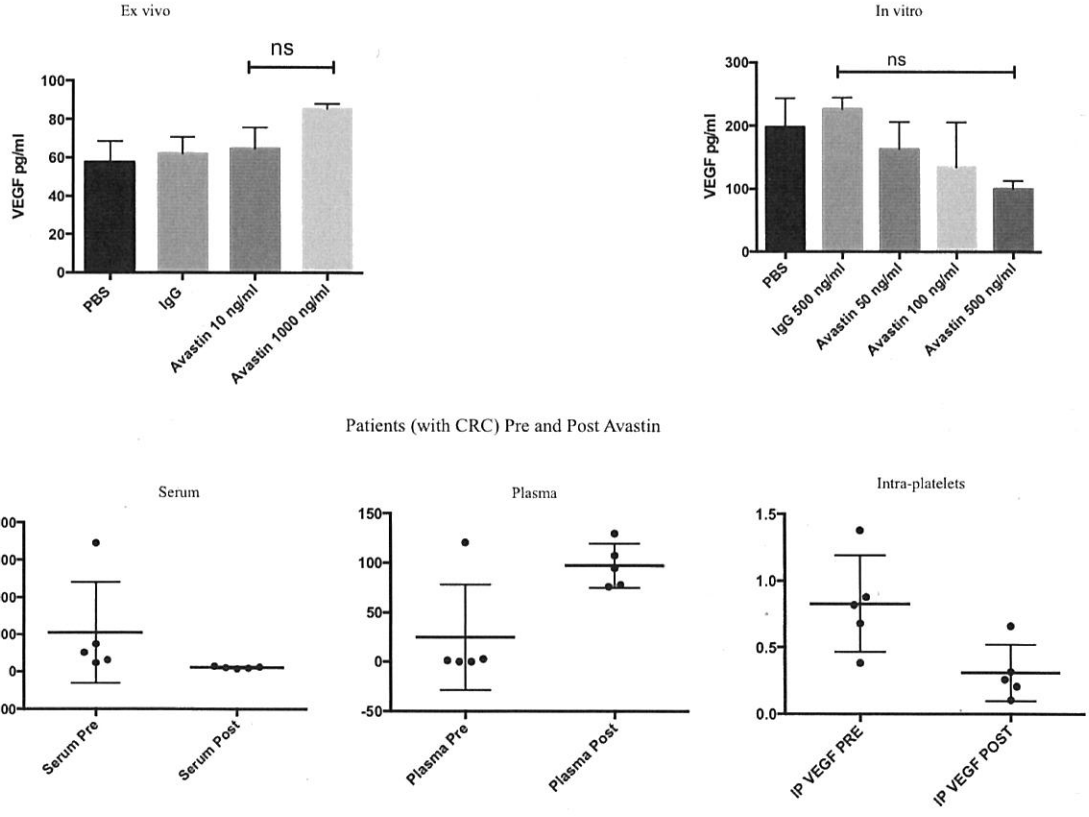
LD, liver dysfunction; HCC, hepatocellular carcinoma;vWF, von willebrand factor

Project 4: Clinical and molecular biological research on novel therapy of CRC by targeting platelet VEGF-A

We used 3 methods to prove our hypothesis: ex vivo, in vitro and in the samples obtained from human colorectal cancer (CRC) patients. At the beginning of this project, we designed a highly standardized protocol for platelet and plasma preparations to ensure the utmost standard. In the first phase, we performed experiments using an ex vivo and in vitro models to examine the effect of bevacizumab on IP VEGF-A neutralization. With different doses of bevacizumab (Avastin®, Genetech), controlled with serum, we found no effect of bevacizumab on intra-platelet VEGF-A concentrations [Fig, 1 a (ex vivo) 1b (in vitro)]. We repeated our ex vivo and in vitro experiments with another potent anti-VEGF-A agent, pegaptanib (Macugen®, Pfizer), which is an RNA-aptamer, substantially smaller in size as compared to the bevacizumab. In our early findings, both bevacizumab and pegaptanib failed to significantly alter the intra-platelet VEGF-A concentrations. These findings suggest that the intra-platelet VEGF-A could influence the impact of bevacizumab treatment, as platelets represent a

major reservoir of VEGF.

The results obtained from human samples were divergent from that of in vitro and ex vivo experiments. VEGF concentrations in serum and intra-platelets were found to drop after systemic administration of bevacizumab, while the VEGF concentrations in plasma were elevated after bevacizumab treatment. Elevation of plasma VEGF after systemic therapy of bevacizumab has been observed in previous few studies. Furthermore, we designed a kit to evaluate different isoform of VEGF, and by the time, we are preparing this report, we are validating the kit and the methods we applied to analyze the isoforms. We found that some specific isoform substantially elevates after bevacizumab treatment, which is highly soluble, free and easily diffusible. This selective elevation of certain isoform of VEGF-A could have a tumor-supporting impact. These results have not been known and could be a game-changing insight on understanding why bevacizumab treatment could not meet the expected efficacy. I am still working one extra month after my tenure to confirm our results and establish its validity before publication. We will submit the further details of our findings to JSPS after validation and publications of our results.



10. Research Presentations during the period of the fellowship

1.) Platelet-derived Growth Factors Predict HCC Recurrence After Partial Hepatectomy in Humans. Bibek Aryal, Toshiaki Shimizu, Jun Kadono, Akira Furoi, Teruo Komokata, Munekazu Yamakuchi,

Teruto Hashiguchi, Yutaka Imoto [62nd Annual Meeting of the Japanese Clinical Laboratory Medicine Kyushu meeting 28th Annual Meeting of the Japanese Society of Clinical Chemistry, Kyushu Branch of the General Assembly, Kurume, Japan 2017-03-04

2.) Intra-Platelet Serotonin After Partial Hepatectomy: A Candidate Predictor of HCC recurrence. Bibek Aryal, Toshiaki Shimizu, Jun Kadono, Akira Furoi, Teruo Komokata, Iwao Kitazono, Munekazu Yamakuchi, Teruto Hashiguchi, Yutaka Imoto [Sixth Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association (A-PHPBA) and the 29th meeting of Japanese Society of Hepato-Biliary-Pancreatic Surgery (JSHBPS), Yokohama, Japan 2017- 06 (07-10)

3.) Qualitative and quantitative platelet crisis in cancer (hepatocellular) recurrence. Bibek Aryal. Yokohama, Japan 2018-03 (11-15)

4.) Perioperative Serum PDGF-BB as a Predictor of Post- Resection HCC Recurrence. Bibek Aryal, Toshiaki Shimizu, Jun Kadono, Akira Furoi, Kentaro Gejima, Teruo Komokata, Chihaya Koriyama, Munekazu Yamakuchi, Teruto Hashiguchi, Yutaka Imoto. 第 11 回桜ヶ丘地区基礎系研究発表会のご案内, Kagoshima, Japan 2018-03-01

5.) Perioperative Serum PDGF-BB Characterizes Patients with Post- Resection HCC Recurrence. Bibek Aryal, Toshiaki Shimizu, Jun Kadono, Akira Furoi, Kentaro Gejima, Teruo Komokata, Chihaya Koriyama, Munekazu Yamakuchi, Teruto Hashiguchi, Yutaka Imoto. Thirtieth Meeting of Japanese Society of Hepato-Biliary-Pancreatic Surgery, Yokohama, Japan 2018-06 (07-09)

6.) Exhaustion of Platelet Kinetics and its Implication in Post-resection HCC Recurrence. Bibek Aryal, Munekazu Yamakuchi, Toshiaki Shimizu, Jun Kadono, Akira Furoi, Teruo Komokata, Kentaro Gejima, Teruto Hashiguchi, Yutaka Imoto. European Society of Medical Oncology (ESMO) Congress. Munich, Germany 2018-10 (19-23)

7.) Depleted PDGF-BB Kinetic in Post-Resection HCC Recurrence- A Risk Factor or Merely an Indicator? Bibek Aryal, Toshiaki Shimizu, Jun Kadono, Akira Furoi, Kentaro Gejima, Teruo Komokata, Chihaya Koriyama, Munekazu Yamakuchi, Teruto Hashiguchi, Yutaka Imoto. 65th Annual Meeting of the Japanese Clinical Laboratory Medicine Scientific Meeting, Tokyo, Japan 2018-11 (16-18)

11. A list of paper published during or after the period of the fellowship, and the names of the journals in which they appeared (Please fill in the format below). Attach a copy of each article if available.

Authors(s)	Title	Name of Journal	Volume	Page	Date	Note
Bibek Aryal, et al.	Predictive Value of Diminished Serum PDGF-BB After Curative Resection of Hepatocellular Cancer	Journal of Oncology	Accepted [In press]	In press	In press	First author
Bibek Aryal, et al.	Therapeutic implication of platelets in liver regeneration - hopes and hues.	Expert Review of Gastroenterology and Hepatolgy	12 (2018)	1219-1228	2018/10/11	First author
Bibek Aryal, et al.	Evaluation of THUNDERBEAT ® in open liver resection- a single-center experience.	BMC Surgery	18 (2018)	1-5	2018/10/16	First author
Bibek Aryal, et al.	Deciphering platelet kinetics in prognostic and diagnostic of hepatocellular carcinoma.	Canadian Journal of Gastroenterology and Hepatology	2018	pp 1-10	2018/06/18	First author
Bibek Aryal, et al.	Post-resection exhaustion of intra-platelet serotonin: Also an indicator of early hepatocellular carcinoma recurrence?	Journal of Cancer	8	3984-3991	2017-10-23	First author

Bibek Aryal, et al.	A 2-stage surgical and endovascular treatment of rare multiple aneurysms of pancreatic arteries.	Annals of Vascular Surgery	40	e9 - 295.e13	2016-11-29	First author
Bibek Aryal, et al.	Conquering the deadly stroke-Perspective on a surgeon's Odyssey.	Annals of Medicine and Surgery	18	14-15	2017-05-03	First author
Bibek Aryal, et al.	Conquering the deadly stroke-Perspective on a surgeon's Odyssey.	Annals of Medicine and Surgery	18	14-15	2017-05-03	First author

12. Awards during the period of the fellowship (Name of the award, Institution, date etc.)

Award: Best Researcher Award. Second Surgery Annual Award Ceromony. Kagoshima University. Graduate School of Medical and Dental Sciences. 2016-12-10

Recognition: Outsanding contribution in reviweing. Elsevier publication. 2017-11-15