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Interaction between MHC Molecules and brother of regulator of imprinting sites (BORIS) in a melanoma cell line

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Abstract Identifying the relationship between the human leukocyte antigens (HLA), MHC class I and MHC class II with tumour antigens expressed in tumour cells may have key points in tumour immunology. If such association occurs it could be relevant to the immune regulation of antigen-mediated immunogenicity. If the intracellular tumour antigen is an immunogenic, it can potentially be presented by MHC molecules to T-cells. A direct consequence of this process is T-cell recognition and thus the development of T-cells capable of targeting the tumour. Key experiments were carried out designed to physically identify the new cancer testis (CT) antigen; BORIS (brother of regulator of imprinting site) and its potential partners. Double immunostaining technique by immunofluorescence method was applied to study a co-localization between MHC molecules (class I and II) and BORIS. Such co-localization studies provide an insight into the intracellular distribution and association of BORIS. This piece of work aims to detect the expression of the tumour antigen of interest (BORIS) in the candidate tumour cell line (MD-MB-435), and to study the co-localisation between BORIS (a potential tumour antigen) with HLA-A,B,C and HLA-DR molecules. The results showed that the co-localization ratio between HLA-DR molecules and BORIS (34.06 %) was slightly higher than the ratio between BORIS and HLA-A,B,C molecules (31.663 %); $P < 0.05$ ***. The co-localization between BORIS and MHC class I or class II molecules could highlight possible interactions between these proteins. Future work on this part of the research should be continued with different tumour cell lines.
Keywords: (BORIS, Cancer testis antigen, MHC molecules, Co-localization, Immunostaining)

التداخل المرتبط بين مركبات التوافق النسيجي والبروتين السرطاني بورز في خلايا سرطان الجلد

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الملخص ان تحديد العلاقة بين مركب التوافق النسيجي (النوع الأول والثاني) ، والذي يعرف بالبروتين المحدد لكريات الدم البيضاء ، مع الأجسام الغريبة السرطانية يمكن أن يكون لها مفاتيح فى المناعة السرطانية . فإذا كان هذا النوع من الإرتباط موجود بينهما ، فقد يكون له علاقة بالتنظيم او التحكم المناعي للمناعة المرتبطة بالجسم الغريب . اذا كان الجسم الغريب السرطاني الموجود داخل الخلايا السرطانية محفز للمناعة فإنه من المحتمل جدا أن يتم عرضه الخلايا التائية عّن طريق مسارات مركبات التوافق النسيجي . أن النتائج المتابعة لهذه العملية تكون تمييز الخلايا التائية للجسم الغريب السرطاني ومن تم تطورها بحيث تكون قادرة على تحديد وتمييز الخلايا السرطانية الحاملة للجسم الغريب والقضاء عليها . وبناء على ذلك تم تصميم هذه الدراسة لمعرفة العلاقة بين بروتين سرطاني جديد يتبع لعائلة تعرف ببروتينات سرطان الخصية ، وبينها وبين مركبات التوافق النسيجي لمعرفة درجة الارتباط بينهما بإستخدام تقنية مناعية وميضية تعرف بالأتحاد المرتبط بين البروتينات داخل الخلايا السرطانية والمحضرة فى صورة خط خلوي سرطاني . أوضحت نتائج الدراسة وجود هذا النوع من الإرتباط بين البروتينات قيد الدراسة ، وأن درجة الإرتباط بين الجسم الغريب ومركب التوافق النسيجي الثاني أعلى بقليل مما عليه مع النوع الأول لمركبات التوافق النسيجي ، وبدلالة معنوية . أن وجود هذا النوع من الإرتباط داخل الخلايا السرطانية يمكن ان يسلط الضوء على وجود تداخل وترابط بين هذه البروتينات له ارتباط مناعي ضد السرطان ، ونقترح متابعة الدراسة على انواع أخرى من السرطان.

الكلمات المفتاحية: بورز ، بروتينات سرطان الخصية ، مركبات التوافق النسيجي ، الترابط البروتيني الموحد ، الصبغ المناعي الوميضي.

Introduction

Multiple cancer antigens have been defined thus far since 1990s [1], of which cancer tumour (CT) antigens have been the main focus of attention. CT antigens are particularly attractive because they have limited expression in cancerous tissues, as they tend to be restricted to normal tissues, mainly in the testis [2]. CT antigens' name is derived from their restricted presence in germ cells of the testes as a normal expression and this characteristic makes them the most attractive target for cancer immunotherapy. They are a multigene family and they have a shared location, frequently, on X chromosome [3]. MAGE-1 (Melanoma antigen-1) was the first tumour antigen that was cloned by Boon and his research group in 1991 [1]. Subsequent cloning of some T cell epitopes along CT antigens was applied to identify new antigens [4-5]. CT antigens have been shown to express in many types of cancer. The wide expression of this group of tumour antigens indicates the importance and the necessity of addressing how they function in normal and tumour cells. Moreover, their potential role as a target for tumours should be investigated. However, although CT antigens have restricted expression in germ cells but some studies have found the expression of some of them in normal tissues as well [6-8]. Due to their restricted expression in cancer cells, CT antigens have been targeted by the researchers for potential use in tumour vaccines, since the development of an antigen-specific cancer vaccine depends mostly on antigens which have restricted expression in tumours and no expression in normal organs. Furthermore, the candidate tumour antigen for a cancer vaccine should have a potent in eliciting a specific humoral B cell and cellular T cell response [2]. Several studies have focussed on the identification of T cell epitopes from CT antigens aimed at generating potent cellular tumour immunity. The successful immune response against tumours demands recognition of epitopes in the tumour cells to create effective T-cell responses targeted at those tumour-associated antigens (TAAs). As a result, their potential role in tumour immunology has been highlighted in the recent studies. A potential immune tolerance against CT antigens has not been reported yet because several studies have showed some of these antigens are immunogenic. Among CT antigens, NY-ESO-1 is the main focus as it is considered the most immunogenic CT antigens known to date. Therefore, more attention has been paid to NY-ESO-1 due to its cellular and humoral immunogenicity. In addition, it's widespread in many tumours makes it a potential candidate for cancer vaccine [9]. BORIS (brother of the regulator of imprinting sites) was recently investigated as a new CT antigen. It was found to be associated with different human cancers including, breast, prostate, ovarian and colorectal cancers, and cells lines [10-11]. In terms of the role of BORIS in cancer cells, there is a controversy on the levels of expression of BORIS on malignancies and whether or not it is a tumorigenic protein. BORIS expression was detected in 77% of endometrial cancers and uterine cancers [11]. It was found that BORIS expressed in the 70% of the breast cancer tissues tested and breast cancer cell lines [12]. The levels of BORIS were correlated with the levels of progesterone and estrogen in the patient with breast cancer. Another important work by the same group found that BORIS was expressed in the blood of the breast cancer patients. Also, the level of BORIS in neutrophils was correlated with the size of the tumour [13]. A recent paper identified BORIS expression in various non-cancerous primary cells including small intestine, colon, liver, ovary etc. Also they identified BORIS to be expressed in various non-tumorous stem cells lines like HEK293 cells along with tumour cell lines [8]. BORIS seems to be expressed in rapidly proliferating cells, and also in undifferentiated stem cells. This also supports the view that cancer is not only a disease of cell proliferation but also a disease of cellular differentiation. A recent study has demonstrated the function of BORIS in cell proliferation and apoptosis in colorectal Cancer. It was found that BORIS expression correlated with colorectal cancer proliferation and BORIS overexpression prompted colorectal cancer cell growth, whereas BORIS knockdown suppressed cell proliferation [14]. Therefore, the potential role of BORIS in tumour immunity should be addressed in a future study. The current study aims to look at the interaction between major histocompatibility molecules and BORIS, as a new member of the CT antigen gene family, in a melanoma cell line and the potential effect on mechanisms of tumour immunity.

Materials and methods

1.Immunostaining: Double immunostaining technique by immunofluorescence method was applied to study a co-localization between MHC (Major histocompatibility) molecules (class I and II) and a new CT antigen (BORIS). Three primary antibodies were used in this assay: mouse anti-human HLA (human leukocyte antigen) -DR (human leukocyte antigen), mouse anti-human HLA-A,B,C and rabbit anti-human BORIS. Indirect immunostaining was applied using the following secondary antibodies: Goat anti-mouse IgG; (immunoglobulin G);(Alexa Fluor 488 with green fluorescence), and Goat anti-rabbit IgG (Alexa Fluor 555 with red fluorescence). Single staining was also performed to detect the expression of BORIS (a candidate antigen) and NY-ESO-1 (a control antigen) in the cytoplasm of MDA-MB-435 cell line, applying flow cytometry (FC) and immuno fluorescence (IF) followed by microscopy.

2.Co-localisation Method : Double staining was carried out for co-localization study, and indirect intracellular immunostaining was applied. Mouse and rabbit anti-human antibodies as primary antibodies of the antigens and secondary antibodies conjugated with Alexa 488 and Alexa 555 were used as mentioned above. MDA-MB-435 cell line was subjected to the study. Confocal microscopy is used for imaging and image

analysis using an algorithm to calculate the percentage of co-localization between HLA-A, B, C and BORIS; HLA-DR and BORIS.

3.Imaging and analysis : Capture of the image and image analysis were performed by using a computational algorithm to determine the percentage of co-localisation between BORIS and MHC molecules. One-way ANOVA was applied for statistical analyses of the data.

Results

1.Expression of BORIS in a melanoma cell line: intracellular expression of BORIS protein in the melanoma cell line (MDA-MB-435) was detected. A high expression of BORIS protein in MDA-MB-435 cell line was observed (Fig.1). The MFI of the BORIS expression is presented in Fig. 2. NY-ESO-1 antigen was used as a control.

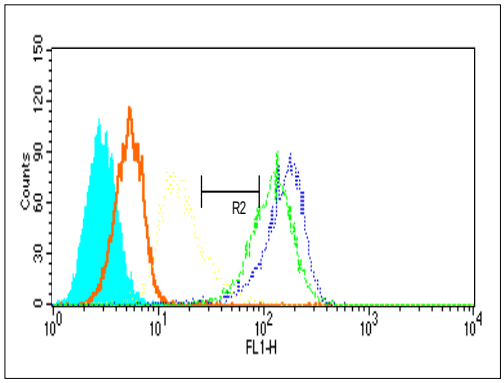

Fig.1: Intracellular expression of BORIS in MD-MB-435 cell line

Indirect immunofluorescence followed by flowcytometric analysis was applied to a melanoma cell line. Rabbit anti-human BORIS mAb as a primary antibody, and goat anti-rabbit secondary antibody conjugated with Alexa 488 were used. The results showed a high expression of BORIS protein (green colour), and NY-ESO-1 protein as a control (blue colour) in the above cell line. Cells without staining (filled histogram) and cells stained with isotype control (orange colour) were used as negative controls.

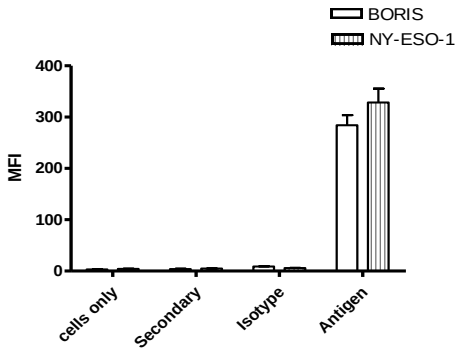

Fig.2: Comparison between the mean fluorescence intensity (MFI)

Comparison between the mean fluorescence intensity (MFI) of the intracellular expression of BORIS proteins in MD-MB-435 cell line. NY-ESO-1 was used as a positive control. The results showed high MFI in the expression of both proteins compared to the controls.

2.Immunostaining for microscopy: MDA-MB-435 tumour cell line was subjected to immunofluorescence staining to view the expression of BORIS. Single immunostaining was carried out using indirect intracellular staining. Rabbit anti-human BORIS mAb was used followed by staining with the secondary antibody (goat anti-rabbit) conjugated with Alexa 488. Fluorescence microscopy is used for imaging. Fig. 3 confirms the expression of BORIS in MDA-MB-435 cell line.

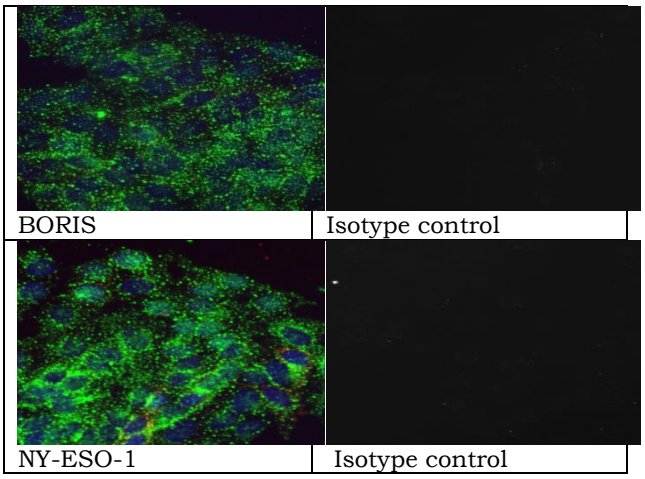

Fig.3: Microscopy image demonstrates the expression of BORIS protein in MDA-MB-435 cell line

Expression of BORIS protein in MDA-MB-435 cell line (left image) compared to the cells with the secondary antibody (right image). NY-ESO-1 was used as a positive control. The cells were cultured in a chamber slide, and grew in 37 ^{0}C incubator overnight. BX41 was used for imaging at 60X magnification.

3.Colocalization between HLA-A, B, C and BORIS:

The cells were stained with both HLA-A, B, C and BORIS primary antibodies. Image acquisition was done using a confocal microscopy followed by image analysis using an algorithm to calculate the percentage of co-localization between HLA-A, B, C and BORIS. Fig. 4 and Fig. 5 present the results of co-localization between both types of MHC molecules and BORIS. High percentage of co-localization was demonstrated; it was summarized in Table (1).

Table 1 Percentage of co-localization study

Triplicates	Co-loc %
HLA-DR	8.463
HLA-ABC	7.338
BORIS	10.336
DR+BORIS	34.06
ABC+BORIS	31.663
isotype con	5.431
secondary Ab only	4.463

Summary of the image analysis of co-localization between both classes of MHC molecules and BORIS. (Co-loc: Co-localization; con: concentration)

4.Colocalization between HLA-DR and BORIS

The same procedure was applied for the study of co-localization between HLA-DR and BORIS in MDA-MB-435 cell line using both primary antibodies of these proteins. The results also showed high percentage of co-localization between HLA-DR and BORIS (Table (1), Fig. 4 and Fig. 5)

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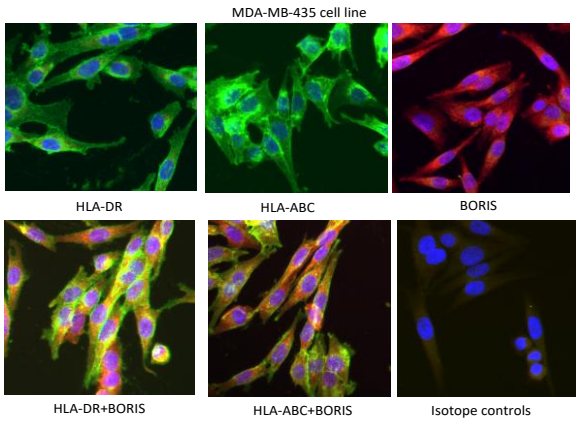

Fig. 4: Co-localization study between both types of MHC molecules and BORIS

Co-localization study between both types of MHC molecules and BORIS in MDA-MB- 435 cell line. The cells were cultured and stained as mentioned previously. The images were acquired using confocal microscope, and analysed to calculate the percentage of co-localization between HLA-A, B, C and BORIS, and HLA-DR and BORIS.

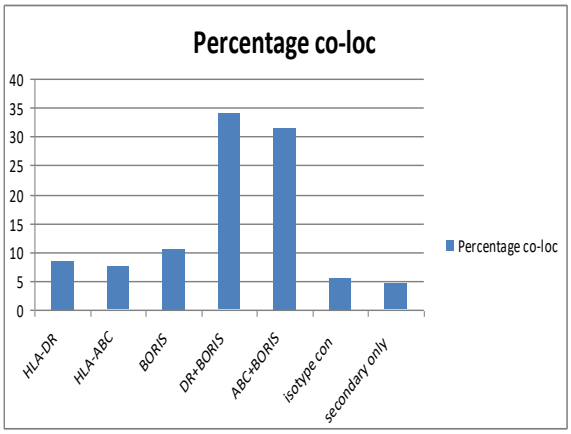

Figure 5 Percentage of Co-localization study

The histogram represents the results of image analysis using an algorithm to calculate the percentage of co-localization between MHC molecules and BORIS. High percentage of co-localization was demonstrated between both types of MHC molecules and BORIS.

Discussion

Identifying the relationship between the human leukocyte antigens, MHC class I and MHC class II with tumour antigens expressed in tumour cells may have key points in tumour immunology. If such association occurs it could be relevant to the immune regulation of antigen-mediated immunogenicity. If the intracellular tumour antigen is an immunogenic, it can potentially be presented by MHC molecules to T-cells. A direct consequence of this process is T-cell recognition and thus the development of T-cells capable of targeting the tumour. Key experiments were carried out designed to physically identify BORIS (Brother of Regulator of Imprinting Site and its potential partners. Such co-localization studies provide an insight into the intracellular distribution and association of BORIS. Co-localization between MHC class-I or MHC class-II with tumour antigens may predict $CD8^+$ (cytotoxic) or $CD4^+$ (helper) T cell epitopes on that antigen, respectively. This will highlight which pathway of antigen presentation can BORIS is engaged (i.e., MHC class-I pathway for $CD8^+$ T cell epitopes or MHC class-II for $CD4^+$ T cell epitopes). Co-localization between NY-ESO-1 and MHC class I and class II molecules can be used as a positive control for this strategy as some MHC class-I- and class-II-restricted epitopes have been mapping on NY-ESO-1 protein, and both of the above antigens belong to the cancer testis family. In addition, a hypothesis for future study has been postulated to subject BORIS as a candidate antigen for mapping immunological T cell epitopes on it.

The key question for this piece of work was: Can a co-localisation between MHC molecules and tumour antigens provide any insights into tumour immunology? The aim of the co-localization study was to highlight the interaction between the MHC molecules as immunological proteins and BORIS (Brother of Regulator of Imprinting Site) as a tumour antigen, and to identify the potential binding of BORIS on MHC molecules. The hypothesis was based on BORIS, an intracellular protein, and whether it could bind to MHC class-I and/or MHC class-II molecules in tumour cells. This aimed to provide an insight into the immune regulation and/or immune response against BORIS.

The critical question is: Why is BORIS engaged in the present co-localization study? The answer would be that BORIS has been found in many cancer cell lines and tumour tissues [11-12]. It is also one of the cancer testis (CT) antigens, which are attractive candidates for aiding immunotherapy. In addition, BORIS is considered a new CT antigen that it has not been mapped for T cell epitopes, and this is the most important reason why it was chosen. By comparison, some other CT antigens have been mapped for T cell epitopes, such as NY-ESO-1 [15-26]. In support of the above reasons, some studies have shown that vaccinating mice with the DNA of mutant murine BORIS and mutated BORIS induced antigen-specific T and B immune responses resulted in inhibited tumour growth and prolonged survival [27-29]. All the above reasons make BORIS a promising tumour antigen for cancer immunotherapy.

More recently, it has been suggested that an effective strategy of vaccination could be to use a

dendritic cell-based vaccine against tumours [30]. This vaccine is based on loading the DCs from mice with a mutated form of BORIS. Those mice were implanted with a metastatic mammary tumour (4T1). Interestingly, the above vaccine strategy induced an effective anti-tumour immunity by inhibiting tumour growth and decreasing metastases. These findings support the idea of using BORIS as a vaccine and studying its effectiveness. Based on the above results, it can be concluded that BORIS could induce cellular immune responses (helper and cytotoxic T cells response) as well as humoral immunity (B cell response).

Interestingly, some studies have indicated that BORIS is expressed very early in malignancy. It has been also suggested that it enhances the expression of other CT antigens such as MAGE and NY-ESO-1 [31-32]. Another study was carried out to examine whether BORIS induces the expression of NY-ESO-1 in lung cancer cells, and it suggested that induction of BORIS could form a novel strategy to enhance NY-ESO-1 expression in pulmonary carcinomas [33]. Additionally, the data from a recent study suggest that BORIS functions as an oncogene in colorectal cancer and highlights the potential clinical applications of BORIS for the treatment of colorectal cancer [14]. These findings support the view that BORIS could be an attractive candidate for developing a vaccine for cancer immunotherapy.

Conclusions

A co-localization study between tumour antigens and MHC molecules could be adopted for determining the possible interactions that occur between them. It could be suggested as a new strategy for mapping immunogenic epitopes (T-lymphocyte epitopes) on tumour antigens. This could serve to predict the potential of peptide-based cancer vaccines for fighting tumours. The results of the co-localisation study between both classes of MHC molecules and BORIS were significant compared to the controls. This suggests that either they are interacting with each other and there are many possible binding sites between them, and also that those binding sites could be immunogenic epitopes, or that it could be relevant to the molecular regulation of MHC molecules by BORIS-mediated tumorigenicity. This kind of study can be applied as a first step for mapping T-cell epitopes on a candidate antigen, as mapping immunogenic epitopes on tumour antigens could make a new contribution to peptide-based cancer vaccines. In conclusion, expression of BORIS on various tumour cells has been shown in some previous studies, and that needs to be expanded to cover other types of tumour and normal tissues. Future work on this part of the research should be continued. Therefore, it can be postulated that BORIS could be an ideal target for cancer vaccines.

Abbreviations and Acronyms

CT: Cancer tumour; BORIS: Brother of regulator of imprinting site, FC: Flow cytometry, IF: Immunofluorescence , MHC: Major histocompatibility , AB: Antibody , IgG: Immunoglobulin G, HLA: Human leukocyte antigens , TAAs: Tumour-associated antigens

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