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INVESTIGATION OF THE PATHWAYS INVOLVED IN THE ACQUIRED RADIORESISTANT EMT6 MOUSE MAMMARY CARCINOMA CELLS TO GAMMA-RAY IRRADIATION: *IN VITRO* AND *IN VIVO* STUDIES

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ABSTRACT

Resistance of breast cancer to radiotherapy is the crucial aspect leading to relapse and low survival rate. Radioresistance is suggested to be linked with epithelial-mesenchymal transition (EMT), a process involved in regulating cancer tissue remodelling, resulting in recurrence and metastasis. The underlying mechanisms in acquiring radioresistance to gamma-ray in mouse mammary cancer cell lines (EMT6) were investigated using both *in vitro* and *in vivo* approaches. EMT6 cells were irradiated with gamma-ray at 2 Gy/cycle to initiate the development of radioresistance *in vitro*. Confirmation of EMT6 cells acquired radioresistance was analysed using migration and clonogenic assays. Next-generation sequencing analysis validated 16 genes of interest (GOI) via real-time polymerase chain reaction (qPCR). The signalling pathways and proteins involved in EMT6 cells acquiring radioresistance were verified by KEGG pathway analysis and western blotting, respectively. $EMT6^{RR\_MJI}$ radioresistance cells were developed from parental EMT6 cells after 8 cycles of fractionated gamma-ray irradiation. This is confirmed by irradiation with gamma-ray at 2, 4, and 8 Gy, which resulted in higher survival fractions and migratory rates of $EMT6^{RR\_MJI}$ compared to parental cells. Six out of 16 GOIs (PD-L1, IL-6, AXL, GAS6, IGFBP4, and APCDD1) were upregulated at the 8^{th} irradiation cycle in $EMT6^{RR\_MJI}$ compared to control. Pathway analysis showed PI3K-AKT and JAK-STAT signalling pathways were the common functional pathways contributing to radioresistance by these six genes. Further investigations focused on PD-1 gene and protein expressions, in which PD-1 is the crucial regulator of PI3K-AKT and JAK-STAT pathways. PD-1 gene and protein expressions were upregulated in $EMT6^{RR\_MJI}$ compared to parental cells ($p<0.05$, $p<0.05$, respectively). The role of PD-1 in radioresistance was further investigated *in vivo*. Mice were divided into 3 groups: Group 1 (control), Group 2 (inoculated with parental EMT6 cells), and Group 3 (inoculated with $EMT6^{RR\_MJI}$ cells). Groups 2 and 3 were further subdivided into 4 subgroups: Subgroup 1 (control, n=6), Subgroup 2 (gamma-ray irradiation, n=6), Subgroup 3 (combination of gamma-ray and Nivolumab, a PD-1 inhibitor, n=6) and Subgroup 4 (Nivolumab, n=6). Mice in subgroups 3 and 4 were further injected with 10 mg/kg body weight Nivolumab at 3 time points. Once the tumour was palpable and visible in all groups, the mice in subgroups 2 and 3 were treated with gamma-ray at 2 Gy per cycle for 8 cycles. Tumour volumes were measured every 2 days. The mice in all groups were sacrificed 5 days post-irradiation and tumour sections were collected to determine the expression of N-cadherin, E-cadherin, PD-1, and PD-L1 using qPCR, while the PD-1 protein was analyzed by western blot and ELISA. N-cadherin and E-cadherin were upregulated and downregulated, respectively, in both tumours. The tumours were confirmed to be derived from proliferative EMT cells. In the parental EMT6, tumour growth in subgroups 2 ($p<0.01$), 3 ($p<0.001$), and 4 ($p<0.01$) decreased compared to control. Furthermore, PD-1 gene expression was higher in subgroup 2 ($p <0.05$) compared to the control group. PD-1 protein expression increased in subgroup 4 ($p<0.05$) but reduced in subgroup 2 ($p<0.05$) as compared to control. For the $EMT6^{RR\_MJI}$ tumour, the growth in subgroup 3 was lower in control ($p<0.001$) group. PD-L1 gene expression decreased in subgroups 2 and 4 compared to $EMT6^{RR\_MJI}$ control ($p<0.05$). PD-1 protein expression was reduced in subgroups 2 and 3 compared to the control group ($p<0.05$). In conclusion, $EMT6^{RR\_MJI}$ acquired resistance to gamma-ray is associated with the upregulation of PD-1/PD-L1 signalling. However, no conclusive evidence suggests Nivolumab inhibits PD-1 expression. Extending the duration of post-treatment is required to elucidate these possible mechanisms, which may provide knowledge for the potential adjuvant treatment during radiotherapy in reducing resistance and recurrence.

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Cancer is a condition in which abnormal cells proliferate out of control and metastasis to other locations. Cancer is the second leading cause of death after cardiovascular disease (Ritchie et al., 2018). Out of 19.3 million new cases diagnosed in 2020, lung (2.21 million) and breast (2.26 million) are the most common types of cancer among men and women, respectively (Sung et al., 2021). Despite extensive research conducted, its aetiology remains poorly understood with the contributing factors not fully discovered.

Breast cancer is the leading cause of death among women in Malaysia and globally (*Cancer Today*, 2020.; Sung et al., 2021). It is characterised by the multiplication of progressive cells leading to the uncontrollable development of tissue masses or tumours in the breast (*Breast Cancer Overview*, 2020.). Benign breast tumours are non-invasive or occasionally precancerous, while malignant tumours can invade and colonise secondary sites (*What Is Cancer?*, 2007). Some malignant tumours developed invasive characteristics enabling them to metastasis from the original site, and migrate through various transporting channels, such as the blood circulation and/or lymphatic system, to initiate secondary site colonisation (Christofori, 2006; Hapach et al., 2019). The metastasis mechanisms involved complicated cell division pathways from the main tumours, invasion, immune monitoring, and control of the tissue microenvironment. Interestingly, epithelial-mesenchymal transition (EMT) is required for metastasis in most malignancies (Williams et al., 2019)

The link between the initiation of breast cancer metastasis and EMT, a condition in which epithelial cell polarity and intercellular cohesiveness are disrupted has been documented (Brabletz et al., 2018). Cell-cell adhesion and cellular polarity are disrupted throughout the EMT process, and modifications in cell-matrix adhesion. EMT is induced in malignancies by hypoxia, cytokines, and growth factors secreted by the tumour's microenvironment, stromal crosstalk, metabolic changes, innate and adaptive immune responses, and anticancer pharmaceutical therapy (Roche, 2018). Interleukin-