Triple Negative Breast Cancer and Synthetic Lethality

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SUMMARY

Triple negative breast cancer (TNBC) is characterized by the lack of expression of estrogen receptors (ER), progesterone receptors (PR), and the human epidermal growth factor receptor 2 (HER2). Despite the recent and extensive search for new therapeutics, few have been proven successful. This review article investigates synthetically lethal interactions within TNBC involving poly(ADP-ribose) polymerase (PARP) inhibitors and the overexpression of the MYC pathway. It also explores the screening for synthetic lethality by utilizing RNA interference, Cas9 libraries, and chemical screens. Limitations of synthetic lethality to treat cancer was also considered. By performing a holistic review of the current known synthetic lethal interactions, conclusions can be made about the implications of this research and the potential for future use in the treatment of cancer patients.

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INTRODUCTION

In 2017, there were an estimated 206,200 new cases of cancer in Canada, and this number continues to rise (Canadian Cancer Society, 2017). With the increase in individuals requiring cancer treatment, the development of more effective and lower risk methods is of great necessity. Currently, cancer treatments impact not only the rapidly dividing cells within the body, but are also proven to be toxic to normal cells, and are therefore characterized as having a low therapeutic index (Kaelin, 2005). Thus, research into novel cancer therapies is of great importance.

In general, the development of carcinogenic tumors is attributed to biological characteristics, referred to as the six hallmarks of cancer. More specifically, this refers to the cell's resistance to death, constant proliferation, ability to induce angiogenesis, capacity to metastasize to other locations within the body, the evasion of growth suppressors, and the presence of replicative immortality (Hanahan and Weinberg, 2011). Each of these six hallmarks are initiated by mutations in genomic DNA and allow for the characterization and classification of carcinogenic tumor cells.

BACKGROUND

TRIPLE NEGATIVE BREAST CANCER

Breast cancer is the most common type of cancer in women. Current treatment methods are usually radiation therapy and chemotherapy, however new treatments are beginning to surface (Li, Uribe and Daling, 2005). Breast cancer is characterized by different combinations of the expression or underexpression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptors 2 (HER2) (Li, Uribe and Daling, 2005).

One important type of breast cancer is triple negative breast cancer (TNBC) and its nomenclature is due to the lack of expression of ER, PR and HER2 within the tumors (Chavez, Garimella and Lipkowitz, 2010). TNBC is a

heterogeneous disease and is known for its distinct metastatic patterns, and lack of targeted therapies (Aysola et al., 2013). Approximately 170,000 of the one million annual worldwide diagnoses of breast cancer are TNBC (Wahba and El-Hadaad, 2015). Using cDNA expression analysis, the majority of TNBC cell lines have been classified as basal or luminal based, with approximately 75% being basal-like (Chavez, Garimella and Lipkowitz, 2010; Wahba and El-Hadaad, 2015). Both Basal and luminal are two of the five main subtypes of TNBC, including luminal A, luminal B, HER2/Neu overexpressing, basal-like (BL1,BL2) and normal-like, each being differentiated by their relative level of expression of hormone receptor genes (Aysola et al., 2013, Xu, Chen and Olopade, 2010). Each subtype has different characteristics and molecular pathways, which is reflected in the manner in which they are treated. Luminal and basal are often classified as being the more aggressive subtypes, making treatment methods more challenging to formulate.

Since patients with TNBC do not contain the target receptors ER, PGR, or HER2, the method of hormone or trastuzumab based therapy is not beneficial (Wahba and El-Hadaad, 2015). This leads to surgery and/or chemotherapy as the only available methods of treatment against the cancerous tumors. Due to the aggressive nature of TNBC, rapid resistance development, and low therapeutic index of chemotherapy, both surgery and chemotherapy are seen to be ineffective treatment methods. Thus, the development of a more effective treatment for TNBC is essential (Chavez, Garimella and Lipkowitz, 2010). Angiogenesis inhibitors, epidermal growth factor receptor targets, Src kinase, and mTOR inhibitors have all been unable to provide feasible data (Nowsheen et al., 2012). However, the concept of synthetic lethality involving PARP inhibitors or the overexpression of the MYC pathway has been proven to be promising methods of therapy with less adverse side effects (Aysola et al., 2013).

OVERVIEW OF SYNTHETIC LETHALITY

Synthetic lethality is when the co-occurrence of two genetic interactions results in cellular or organismal death (Nijman, 2011). In terms of cancer, an interaction is considered synthetically lethal when a mutation occurs within a cancer specific protein and the tumor cell requires the activity of the synthetic lethal partner for gene viability (O'Neil, Bailey and Hieter, 2017). Within the field of synthetic lethality, there are three main types that can create a feasible interaction. The types include a double mutation resulting in loss of cell viability, the mutation of one gene coupled with the induced inhibition of another, and finally synthetic dosage lethality, where the overexpression of a gene and induced inhibition of another causes cell death (Figure 1) (O'Neil, Bailey and Hieter, 2017).

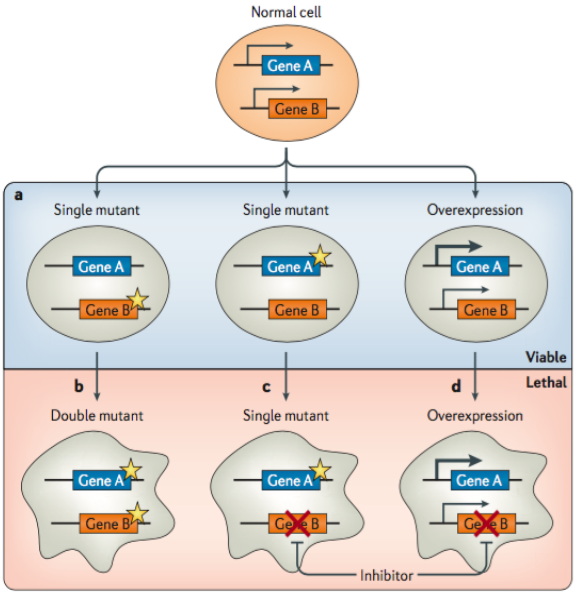

Figure 1: A simplified diagram showing various scenarios of synthetic lethal interactions. Mutation of either gene A (blue) or B (orange) is viable, or the overexpression of both (Case A). In comparison, a mutation in (Case B), the inhibition (Case C) or the inhibition and overexpression (Case D) of the genes causes synthetic lethality (O'Neil, Bailey and Hieter, 2017). Continual activation of the downstream mutant can result in drugs of the target being rendered ineffective. Similarly, if the mutant is upstream of the target, resistance to drugs can be attained by a mutant bypassing a protein in the signalling pathway, such as ERBB2 activating AKT3 rather than PI3K (Miller, Goulet and Johnson, 2016).

The field of synthetic lethality has been proven to have potential as it allows for the targeting of non-druggable cancer mutations after the identification of the second mutation site or inhibitor synthetic lethal target. Currently the concept of synthetic lethality has been proven to be very effective within budding and fission yeast, engineered cells, and transgenic mouse models (Kaelin Jr, 2005; Ooi et al., 2006; Yang et al., 2010). Since a vast amount of information is known about these organisms and the manner with which a synthetically lethal interaction can be screened for, synthetic lethal interactions have been easy to identify (O'Neil, Bailey and Hieter, 2017). In terms of human cell cultures, however recent advances are being made in the field of RNAi and CRISPR technology to increase the ease of finding synthetic lethal interactions.

METHODS

Preliminary information was obtained by searching Google Scholar, and peer reviewed papers were used to provide the information given in this paper. While searching for articles, the terms used varied depending on the section. Often both the terms "synthetic lethality" and "triple negative breast cancer" were used throughout in order to direct the research to the specific method of treatment and breast cancer. Otherwise the terms: "PARP inhibitors", "MYC pathway", "RNA interference", "CRISPR-Cas9", and "chemical screens" were used for the various sections. The pathways that are discussed in the paper were chosen based on the amount of research being conducted on the particular pathway. A pathway was considered as a lead if the pathway was used by cancerous cells, and if the pathway selected had a role in the nucleic function of a cell.

PATHWAYS ILLUSTRATING SYNTHETIC LETHALITY POTENTIAL

In terms of TNBC, two known synthetically lethal interactions include PARP inhibitors and the MYC pathway (Lord and Ashworth, 2017; Yang et al., 2010). PARP inhibitors are an example of the mutation of a gene coupled with the inhibition of another, while the MYC pathway is an example of synthetic dosage lethality.

PARP INHIBITORS

Of great interest to the field of synthetic lethality is the poly(ADP-ribose) polymerase (PARP) inhibitors, as they were the first clinically approved drug type to exploit the use of synthetic lethality (de Lartigue, 2013, Lord and Ashworth, 2017). The DNA damage response (DDR) is the process with which cells defend themselves against DNA damage (Lord and Ashworth, 2017). PARP is responsible for the detection of the DDR, and more specifically, the associated single-strand DNA breaks (Lord and Ashworth, 2017). Single stranded breaks (SSBs) cause conformational changes to the damaged DNA and create a binding site for PARP (Lord and Ashworth, 2017). Once bound, a conformational change occurs, causing the activation of PARP's catalytic function (Lord and Ashworth, 2017). This catalytic activity causes the PARylation of PARP proteins, initiating DNA repair by attracting DNA repair effectors and altering the chromatin structure (Lord and Ashworth, 2017). After the repair is complete, autoPARPylation occurs, and PARP is released (Lord and Ashworth, 2017).

Within cancerous tumors, PARP inhibitors (PARPi) stimulate nicotinamide adenine dinucleotide (NAD), the enzyme cofactor, and inhibit PARylation (de Lartigue, 2013). When inhibition occurs, PARP is no longer able to be released through autoPARylation, causing the buildup of PARP in a process known as PARP trapping (de Lartigue, 2013). This prevents DNA replication initiation, and homologous recombination repair (HRR) is required to restore regular nucleic function to the affected cell (Figure 2) (Lord and Ashworth, 2017)Within the PARP pathway, the synthetic lethal interaction is a result of mutations within the *BRCA1* and *BRCA2* genes (Lord and Ashworth, 2017). Mutations in *BRCA1/2* result in an increased chance for the development of breast and ovarian cancer (Mehrgou and Akouchekian, 2016). These genes are required for HRR and without the presence of HRR, alternative methods of DNA repair thrive, although these methods can be the cause of numerous DNA mutations (Lord and Ashworth, 2017). In combination with the mutations in either *BRCA1* or *BRCA2* and induced PARP inhibition, the synthetically interaction can be used to prevent the repair of DNA breaks and induce tumour cell death (Lord and Ashworth, 2017). Thus, PARP

inhibition is an example of a mutation (*BRCA1/2*) and inhibition, causing synthetic lethality.

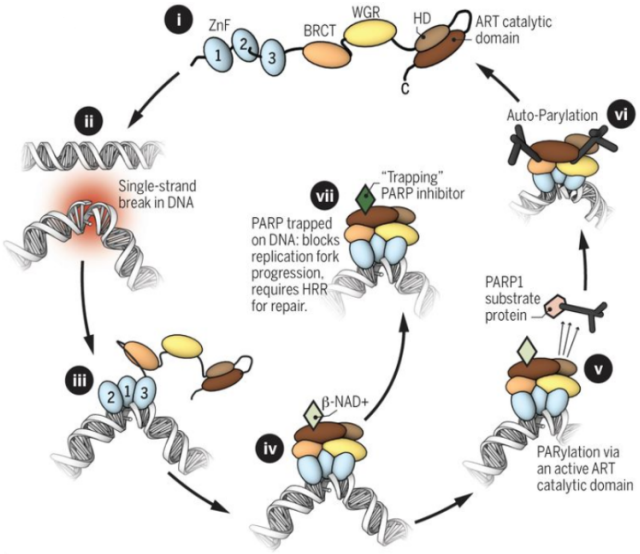

Figure 2: The PARP cycle. PARPi disrupts the cycle in its fourth stage, causing the repair process to halt (Lord and Ashworth, 2017).

THE MYC PATHWAY

In the pathogenesis of numerous human cancers, the *myc* proto-oncogene is important within multiple signal transduction pathways (Dang, 2012). *Myc* is a regulatory gene coding for transcription factors required for many cellular processes, such as ribosome production, metabolism, and the cell cycle - all of which govern the growth rate of the cell (Hsieh and Dang, 2016). Alterations to the regulation of *myc* can cause the pathways to bypass normal control checkpoint mechanisms (apoptosis and senescence), ultimately contributing to the uncontrollable cancerous cell growth commonly observed in tumors (Gabay Li and Felsher, 2014). Within cancerous cells, the myc pathway becomes a means for controlling the induction of stemness and blocking cellular senescence and differentiation (Gabay Li and Felsher, 2014). It is also responsible for controlling the microenvironment of tumors, the activation of angiogenesis, and suppression of the host immune response (Gabay Li and Felsher, 2014).

On a molecular level, the myc transcription factor contains a basic helix-loop-helix zipper (bHLHZ) motif and is regulated by the binding of another bHLHZ protein, termed MAX (Figure 3) (Horiuchi et al., 2012). MAX can either bind to an activator such as Myc, or a repressor such as MXD1-4. MXD1-4 competes with myc for MAX binding. Within TNBC, myc is overexpressed and the regulation of *myc* expressions is altered (Fallah et al., 2017, Horiuchi et al., 2012). More specifically, the Myc activators are up-regulated while the MXD1-4 is significantly down-regulated. This reduces the competition, leading to the overexpression of the myc pathway (Horiuchi et al., 2012). In most cancers, myc expression is deregulated, although in breast cancer it is overexpressed in 30-50% of high-grade tumors (Fallah et al., 2017). In order to treat cancer progression, inhibition of this pathway at a specific target is a potentially viable therapeutic route. Within TNBC, a direct target of Myc remains unknown. Currently, research is being conducted on potential targets that can be paired with the overexpression of MYC to produce a synthetically lethal interaction (Fallah et al., 2017).

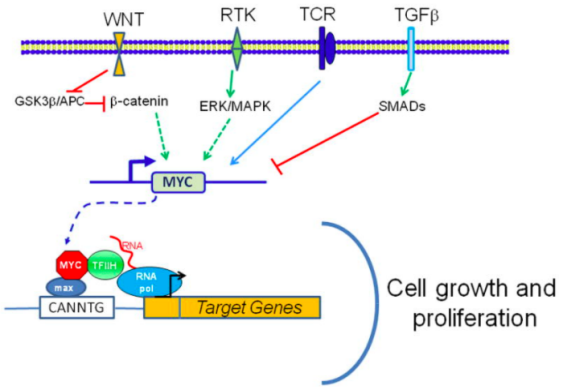

Figure 3: The MYC proto-oncogene is found within many receptor signal transduction pathways, a few of which are depicted in the figure. The MYC gene codes for the transcription factor Myc, and after binding with MAX and the target DNA sequences regulation of transcriptional genes important to cell growth and proliferation occurs (Dang, 2012).

In the exploration of synthetic lethality interaction, a few possible targets have been identified through the use of varied screens. Synthetic lethality between the overexpression of the MYC pathway and the inhibition of cyclin-dependent kinases (CDK) has been illustrated in engineered cells and mouse models (Goga et al., 2007). CDKs are

essential in the progression to the next stage of each step in the cell cycle; numerous varieties exist for the different stages of the cycle. For instance, Cdk1 is a subfamily responsible for centrosome maturation and separation, chromosome condensation, and entry into the mitotic phase after the breakdown of the nuclear envelope (Malumbres, 2014). Extending this idea to TNBC, it has since been observed that MYC overexpression and the inhibition of CDK successfully halts the growth of TNBC within xenografts (cells from patient tumour transported into immunodeficient mice). More specifically, through the use of a purvalanol A and dinaciclib CDK inhibitors a synthetic lethal interaction has been found in xenografts (Horiuchi et al., 2012).

In cells overexpressing Myc as a result of the inhibition of Cdk1 using synthetic lethality, an increase in a proapoptotic protein, Bim of the Bcl-2 family, has been observed (Bcl-2 interacting mediator of cell death) (Horiuchi et al., 2012). BCL-2 family members control an initial step within apoptosis, the permeability of the outer mitochondrial membrane (Shamas-Din et al., 2013). Horiuchi et al. (2012) observed that Bim was upregulated after MYC overexpressing cells were treated with purvalanol A. Thus, with an upregulation of Bim in cancerous cells, an increase in apoptosis occurs (Horiuchi et al., 2012).

SYNTHETIC LETHAL INTERACTIONS-SCREENS

Once a pathway that cancerous cells are dependent on is targeted, the next step in treatment development is to discover a mode to disrupt its function through synthetic lethality, while leaving healthy cells relatively unharmed. This can be done by performing screens for interactions that cause the cancerous cells to die. This process is known as screening (Barbour and Xiao, 2006). For TNBC, there are three main types of screens that are of interest for human cells: RNA interference, CRISPR-Cas9, and chemical (Nijman, 2011).

RNA INTERFERENCE (SIRNA)

RNA interference (RNAi) is a biological response to double-stranded RNA (dsRNA) that allows a cell to silence a gene by inhibiting RNA translation. This is done by using short interfering double stranded RNA (siRNA) (National Center for Biotechnology Information, 2017). One strand of siRNA then binds to a protein-coding mRNA complex that has a complementary nucleotide sequence to the siRNA. This complex binds to mRNA, which blocks translation and thus silences the gene (Carthew and Sontheimer, 2009; National Center for Biotechnology Information, 2017). RNAi has been used to induce synthetic lethality in multiple cancers (Dai, Fang and Roth, 2009). More specifically, it has been used to target EGFR in lung cancer, and HER2 in breast cancer.

To screen for synthetic interactions between genes, the precursor of siRNA - specifically pooled shRNA libraries - are typically used (Diehl, Tedesco and Chenchik, 2014). This allows multiple shRNAs to be tested at the same time (Kampmann et al., 2015). To perform a dropout viability shRNA screen, a library of shRNA is introduced to a population of live cells by a single large-scale transduction, and the shRNA is integrated into the genomic DNA of these cells (Diehl, Tedesco and Chenchik, 2014). The transduced cells are then left to incubate for at least six doublings, which allows the shRNA to be expressed inside the cells, and thereby cause the cells to display the associated phenotype of the gene in question in the presence of potentially protein-inhibiting shRNA (Kampmann et al., 2015). After the growth period is complete, high-throughput sequencing is used to determine the relative shRNA levels in the cell population (Diehl, Tedesco and Chenchik, 2014). If there are shRNAs that are underrepresented in the cell population, it is because those shRNAs killed or inhibited the growth of the cells. These shRNAs can then be considered hits for potentially viable drugs, and their mechanism of action can be elucidated.

CRISPR-CAS9

Clustered regularly interspaced short palindromic repeats - CRISPR associated protein 9 (CRISPR-Cas9) is a genome-editing system that is used to introduce DNA double-strand breaks (DSBs) at a genomic area of interest (Sánchez-Rivera and Jacks, 2015). The CRISPR-Cas9 system is a target-specific single-guide RNA (sgRNA) used in conjunction with the Cas9 endonuclease (Dhanjal, Radhakrishnan and Sundar, 2017). The sgRNA, bound to the Cas9 enzyme, binds to the specific sequence complementary to the sgRNA in the host genome (Redman et al., 2016). After the specific sequence is found, Cas9 cleaves the DNA at that location, introducing a DSB (Sánchez-

Rivera and Jacks, 2015). This DSB is usually repaired by non-homologous end joining (NHEJ) (Sánchez-Rivera and Jacks, 2015). NHEJ repairs the DSB without using template DNA as a reference for the correct genome sequence (Sánchez-Rivera and Jacks, 2015). This causes the repaired DNA to have a higher chance of random insertions and deletions – called indels (Ma et al., 2017). These cause a frameshift that disrupts gene function (Dhanjal, Radhakrishnan and Sundar, 2017). If this gene was integral to the cell's survival, the cell will die (Sánchez-Rivera and Jacks, 2015). This is how a lethal interaction is induced with Cas9. When the interaction is found, the gene can then be used as a target for developing inhibitory drugs to recreate the interactions.

CHEMICAL SCREENS

Another way to test for synthetic lethality is to utilize chemical screens. In this method, the goal is to find chemical leads that can be further developed into anticancer drugs that induce a synthetically lethal interaction (Dahlin and Walters, 2014). Chemical screens are the preferred screening method by some researchers since they can lead to the development of a drug faster than other screens, since a high amount of chemicals can be screened in a short amount of time compared to other types of screens (Barbour and Xiao, 2006). There are two methods that can be used for chemical screens: high throughput screens (HTS) and fragment-based screens (Dahlin and Walters, 2014).

In HTS, the goal is to screen as many molecules as possible in an attempt to identify active compounds that have potential therapeutic activity (Bressan, 2014). HTS are executed in an assay format. Depending on the setup, there are many detection methods to validate the efficacy of the molecules (Wildey et al., 2017). After a screen, there can be multiple molecules identified that may interact with a desirable target (Bleicher et al., 2003). These are narrowed down to a smaller number of molecules that will continue in the drug discovery process (Gupta et al., 2009). The selected molecules are optimized for qualities that function best and a select few will then go on to clinical trials for further drug development (Bleicher et al., 2003).

The utilization of fragment-based screens has increased in popularity since its conception in 1996 (Rees et al., 2004). Fragments are defined as organic molecules which have a low molecular weight, are moderately lipophilic, and are highly soluble (Kumar, Voet and Zhang, 2012). The concept of fragment-based screening is simple – small fragments are put through a screen, and molecules that interact with the target are noted (Kumar, Voet and Zhang, 2012). The small molecules are then combined to create larger ones that can greatly inhibit their target (Rees et al., 2004). The size restriction of the fragments decreases the number of molecules that are screened compared to HTS, with a maximum of a few thousand molecules in one screen (Rees et al., 2004). The interactions are then quantified and validated using nuclear magnetic resonance (NMR) spectroscopy, since NMR chemical shifts are sensitive to ligand binding, and compound interference can be solved with spectral editing to isolate only the spectrum corresponding to the ligand binding (Hajduk and Greer, 2007; Wang et al., 2003). Another advantage of fragment-based screens is that a high proportion of the atoms in the fragment directly interact with the target allowing each fragment to efficiently bind to it (Hajduk and Greer, 2007; Rees et al., 2004).

DRAWBACKS OF DISCUESSED TECHNOLOGIES

As illustrated above both the PARP and MYC pathway have known successful synthetically lethal interactions, thus the number of molecules that need to be screened for potential synthetically lethal interactions decreases. Although ideal for cancer treatment due to selective cell death towards tumor cells, a limitation of synthetic lethality is that it is dependant on a singular pathway. In the case that the cancer being treated develops a mutation allowing the bypass of the exploited pathway, then the drug can become ineffective (Dai, Fang and Roth, 2009). Not only can the cancer cells develop an additional mutation to resist the drug but also they can potentially develop an alternative pathway by amplifying the targeted gene (Pao et al., 2005; Gorre, 2001). Upon further investigation of this synthetic lethality these limitations would have to be further explored in order to determine the potential risks involved in using this method in the treatment of TNBC.

LIMITATIONS

One limitation in the methodology was the lack of anticancer drugs that utilize synthetic lethality as their mode of mechanism. While researching, there was only one FDA approved drug that used synthetic lethality. Due to the substantial amount of research on drugs that are still in the developmental stages, most of the data that may have been beneficial for our research, was unavailable to access. For future steps, a meta-analysis can be conducted on the data available to directly compare the current potential drugs showing synthetically lethal effects. Comparisons could be made on their adverse side effects and overall effectiveness for treatment of TNBC in attempt to propose the most beneficial drug.

CONCLUSION

If a synthetic screen is found to be viable, the molecule that is found can then be further developed into a drug to help combat TNBC. When analyzing all aspects of synthetic lethality and the current knowledge of successful interactions, it can be assumed that there are more lethal interactions that have yet to be discovered.

This progressing shift in traditional cancer treatment raises the chance of survival for many cancer patients. Treatments based on findings of synthetic lethality may not be widespread currently, but it opens a door for new, more specific, cancer treatments.

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AUTHOR CONTRIBUTIONS

R.A. wrote about TNBC, the MYC pathway in TNBC, RNA interference and contributed to the conclusion of the paper. M.O. wrote about synthetic lethality, the PARP pathway, CRISPR-Cas9 and chemical screens. Both Rachel and Maryanne conducted preliminary research on triple negative breast cancer and synthetic lethality, as well as combined the research from the various sections to write the future directions section.

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