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14. ABSTRACT
To define the effects of DMF in preventing recurrence in a xenograft models of ER+ breast cancers (Specific Aim 1), we established xenografts in mice and treated them with vehicle, DMF, Tamoxifen, or DMF+Tamoxifen for 4 weeks. Tumors were excised, RNA was isolated and RNA transcriptomic analysis was conducted. Among the top pathways regulated by both DMF+Tamoxifen were related to immune, host defense response and interferon signaling. We conducted simultaneously chemoproteomics to identify global succination targets of DMF in this setting (Specific Aim 2) and among those targets, covalent modification of IRF9 is currently being investigated for its relevance to interferon signaling and its impact on tumor recurrence. In summary, two complementary approaches, RNA transcriptomic analysis and chemoproteomics, are converging on identifying the IRF9/interferon signaling as critical to tumor recurrence and a therapeutic target of DMF in breast cancer.

15. SUBJECT TERMS
DMF, Recurrence, ER+ breast cancer, Interferon signaling, Tamoxifen tolerance.

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Introduction

We have reported that co-treatment with DMF+Tamoxifen effectively prevented tumor recurrence compared to Tamoxifen treatment alone [1]. DMF as a small molecule is expected to covalently modify target proteins via succination of protein thiols. Therefore, by identifying the succination reactome of DMF in the breast cancer landscape, new critical drivers of tumor recurrence will emerge. In turn, this mechanistic insight will be harnessed therapeutically to prevent tumor recurrence.

Body

1. RNAseq transcriptomics and bioinformatic analysis in DMF+Tamoxifen treated xenograft tumors. Xenograft tumors were established from ER+ MCF-7 breast cancer cells in athymic nude mice treated with vehicle/None, DMF, Tamoxifen, or DMF+Tamoxifen for 4 weeks. After treatments were completed, tumors were excised and RNA was isolated and submitted for RNAsequencing analysis. 2751 genes were selected with p<0.01, fold>1.4 for any of the 3 comparisons (DMF, Tamoxifen, DaMF+Tamoxifen) with None. Genes were centered on the average of the None group and underwent supervised clustering (Fig. 1).

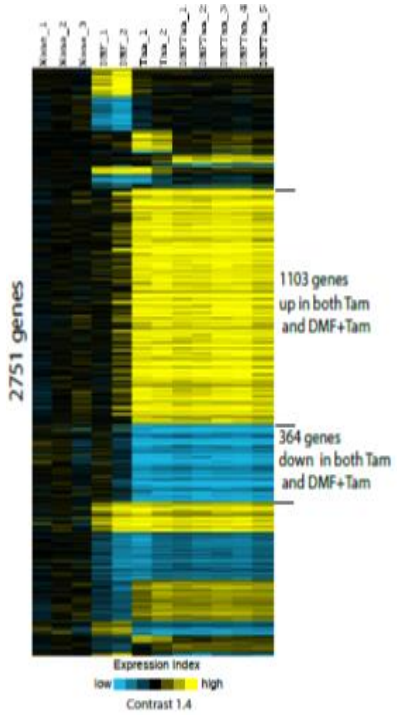

Figure 1. Supervised cluster analysis of top differential regulated genes from four treatment groups.

Given that the commination treatment with DMF+Tamoxifen effectively prevents recurrence [1], we focused on this treatment group. The top enriched pathways among genes upregulated or suppressed by DMF+Tamoxifen treatment are listed in Fig. 2. A number of these top pathways implicate immune and defense response highlighted in red (Fig. 2). This was surprising given that this xenograft model relies on immune-compromised mice. Never the less, these mice lack T-cells but other components of the tumor microenvironment can contribute to tumor recurrence. Particularly, the interferon (IFN) pathway may be relevant to tumor progression and recurrence.

2. IFN signaling in tumorigenesis. IFN signaling encompasses the mechanism through which interferons (IFNs, type I-III) are made and released by the host cell in response to a viral infection. This may explain the 'Defense Response to Virus' signature observed (see table in Fig. 2). IFNs are cytokines that bind their corresponding cell surface receptor, allowing the JAK-STAT (Janus Activated Kinases-Signal Transducer and Activator of Transcription) signaling cascade to initiate. Type I IFNs, such as IFNα/β, bind their cognate IFN-α/β receptor (IFNAR) complex, which is associated with tyrosine kinases. The activation of both JAK1 (Janus kinase 1) and

TYK2 (Tyrosine Kinase 2) results in the downstream phosphorylation of both STAT1 and STAT2. The

Category	Term	Count in Selected Genes	Count in Total Population	P-value
GO	cilium	83	308	5.63E-26
GO	cilium organization	58	215	1.62E-18
GO	motile cilium	40	121	1.69E-16
GO	cilium assembly	52	196	2.12E-16
GO	cilium movement	32	84	1.73E-15
GO	microtubule-based movement	62	280	3.32E-15
GO	plasma membrane bounded cell projection assembly	63	290	5.15E-15
GO	microtubule-based process	104	645	2.27E-14
GO	plasma membrane bounded cell projection	180	1398	4.04E-14
GO	cell projection assembly	63	303	4.45E-14
GO	cell projection	187	1477	5.9E-14
GO	axonemal dynein complex assembly	16	27	4.58E-12
GO	cell projection organization	108	740	5.19E-12
GO	movement of cell or subcellular component	154	1226	2.61E-11
GO	ciliary basal body	34	129	4.14E-11
GO	axoneme	27	86	5.37E-11
GO	plasma membrane bounded cell projection organization	83	553	4.34E-10
GO	microtubule-based protein transport	20	65	2.47E-08
GO	protein transport along microtubule	20	65	2.47E-08
GO	motile cilium assembly	11	20	3.23E-08

Category	Term	Count in Selected Genes	Count in Total Population	P-value
GO	response to biotic stimulus	76	897	3.45E-16
GO	response to external biotic stimulus	73	876	3.45E-15
GO	response to external stimulus	95	1394	4.78E-14
GO	defense response	64	850	1.91E-11
GO	response to other organism	56	701	4.17E-11
GO	immune response	55	718	3.06E-10
GO	defense response to virus	24	174	6.54E-10
GO	response to organic substance	104	1923	2.97E-09
GO	immune system process	96	1730	3.87E-09
GO	response to virus	28	254	4.52E-09
GO	immune effector process	56	803	6.46E-09
GO	interferon-gamma-mediated signaling pathway	14	65	7.55E-09
GO	defense response to other organism	43	540	9.73E-09
GO	response to chemical	120	2389	1.07E-08
GO	cellular process	429	12479	2.47E-08
GO	response to lipid	48	666	3.09E-08
GO	response to stimulus	188	4384	4.98E-08
GO	negative regulation of viral life cycle	14	79	1.04E-07
GO	response to oxygen-containing compound	69	1197	2.17E-07
GO	regulation of viral life cycle	18	141	3.01E-07

Figure 2. Enriched GO terms for genes upregulated with DMF+Tamoxifen treatment (left panel) and for genes downregulated by DMF+Tamoxifen treatment (right panel).

phosphorylated STAT1-STAT2 heterodimer associates with a third transcription factor, interferon regulatory factor 9 (IRF9), leading to the formation of the IFN-stimulated gene (ISG) factor 3 (ISGF3) complex. The ISGF3 complex then translocates to the nucleus and binds DNA sequences referred to as IFN-stimulated response elements (ISRE) within the ISG promoter regions, leading to ISG transcription. While IFN signaling plays a critical role during viral infections, emerging data suggest an important role for IFN in tumorigenesis through modulation of immune surveillance. Independent of viral infection, both IFNs and ISG expression have been found in human tumors. While the effects of IFN signaling and ISG phenotypes vary in cancer biology, it remains clinically relevant to gain a deeper understanding of IFN signaling regulation in this context.

3. IRF9 transcription factor in the IFN pathway is covalently modified by DMF. The chemical biology approach of iso-TOP-ABPP profiling[2] was conduced in tumor lysates treated with DMF. Using this methodology 18,000+ cysteines on 6,000+ proteins were quantified, and 23 cysteines showed >=75% decrease of signal upon DMF treatment (DMF vs DMSO vehicle control) and 229 cysteines showed >=50% decrease of signal. Please refer to the table in Fig. 3 for a full list of DMF succination targets identified in xenograft tumors showing significant inhibition by DMF. Importantly, top hits are site-specific and include previously discovered functional sites such as the IRAK4_C13 proteomic hot spot for DMF in Cal-1 cells published by our collaborator [3]. IRF9 is a top succination target of DMF under these experimental conditions and belongs to the IFN signal transduction described above, suggesting that DMF inhibits IFN signaling in tumors through succination of IRF9. This hypothesis is currently under investigation in our lab. This data raises another important question – what is the role of IRF9 specifically in breast tumor recurrence? This is also currently under investigation in our lab.

4. Both DMF and Tamoxifen regulate IFN signaling and ISGs expression. IFN signaling was identified from the combination treatment with both DMF and Tamoxifen. The 14 IFN-stimulated gene (ISGs) identified in tumors that are suppressed by the combination treatment (see table in Fig. 2) were examined *in vitro* to identify the

relative contribution from each treatment alone. MCF-7 cells were stimulated with IFNy to enhance ISG transcription and the inhibitory effect of DMF alone, Tamoxifen alone, or the combination of DMF+Tamoxifen treatment is measured. We find that a number of ISGs are inhibited by DMF, but not by Tamoxifen as exemplified by LGALS9 expression (Fig. 4A), while other ISGs are inhibited by Tamoxifen but not DMF, as exemplified by IFITM1 expression (Fig. 4B). More importantly, both LGALS9 and IFITM1 are effectively inhibited by the combination treatment DMF+Tamoxifen. This data may highlight the importance of both drugs in the tumor recurrence setting. Inhibition of ISG expression by DMF may occur through succination of IRF9 which is currently being investigated (see Section 3). On the other hand, how does Tamoxifen inhibit IRF9/IFN signaling to result in reduced expression of ISGs? We find that Tamoxifen (4-hydroxytamoxifen, 4OHT) inhibits the transcript levels of IRF9 itself, which may result in attenuation of IFN signaling and ISGs expression (Fig. 4C). In summary, both DMF and Tamoxifen are required for effective inhibition of IFN signaling and expression of ISGs. DMF may work through inhibition of IRF9 via covalent modification, while Tamoxifen may work through transcriptional regulation of IRF9 mRNA expression. Elucidating the role of the IFN-IRF9-ISG axis in ER+ breast cancer recurrence has important implications for the biology of tumor recurrence and may lead to better therapeutic strategies to block tumor recurrence.

Site	Quantified in _ data sets	% inhibition DMF vs DMSO	Cysteines quantified in one protein	Cysteine showed >=50% decrease of signal in the same protein	% inhibition DMF vs DMSO
IRF9_319	2	90	2	1	70.5
HELZ_101	1	89	8	1	Unknown
POLRMT_726	2	87	8	1	57.5
IRAK4_13	2	86	1	1	77
SYNE2_2993	1	84	22	4	67
CDKN3_39	2	82	1	1	65
THNSL1_277	2	82	8	3	Unknown
SYNE2_2994	1	81.5	22	4	74
CHRAC1_55	2	81	1	1	57.5
BIRC2_45	2	81	2	1	62.5
CHMP1A_44	2	78	1	1	58.5
ADO_18	2	78	2	1	67
PRKDC_4045	2	78	37	1	65
SMC2_800	2	77	5	2	60.5
CCDC61_418	1	77	1	1	Unknown
USP7_315	2	77	9	1	54
ICE1_2094	2	77	7	1	70
UACA_980	2	77	5	1	65
KEAP1_151	1	77	12	1	63
AP4E1_1119	2	76	2	1	61
RBM12B_204	2	75	1	1	49.5
HSPBP1_204	2	75	6	1	71
ZMYM1_989	1	75	3	1	47

Figure 3. Top hits for DMF succination targets in xenograft tumors.

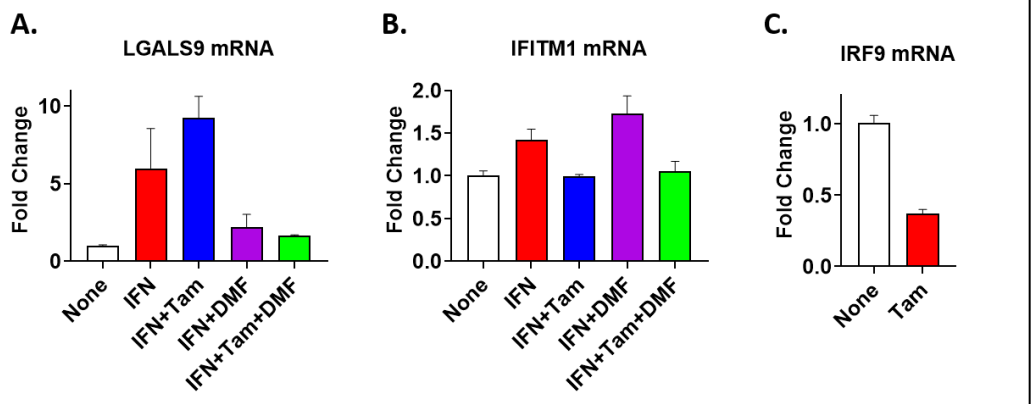

Figure 4. Regulation of ISGs (LGALS9 and IFITM1) and IRF9 expression by DMF and Tamoxifen. (A-B) MCF-7 were treated with IFNy to stimulate IFN signaling and DMF (50μM) or Tam (4OHT, 1μM) were added for 24hrs. (C) MCF-7 grown in estrogenized media were treated with Tam (4OHT, 1μM) for 24hrs. Gene expression is measured by RT-QPCR.

Key Research Accomplishments

- RNAseq and chemoproteomics in xenograft tumors from treated mice are completed.
- These two complementary approaches identified the IFN-IRF9-ISGs axis modulated by our treatments and may be implicated in tumor recurrence.

Reportable Outcomes

1. Our first manuscript on identifying ZNF217 as a druggable target relevant to endocrine resistance and recurrence (see previous year's report) is in preparation for submission.
2. We have made progress in identifying critical signaling pathways and druggable targets of DMF driving tumor recurrence.
3. Ongoing efforts are focused establishing the regulatory mechanisms by both DMF and Tamoxifen at inhibiting IFN-IRF9-ISG axis to prevent tumor recurrence, and elucidate how IRF9 contributes to tumor progression and recurrence.

Conclusion

Our progress is on track and follows the approved SOW.

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Appendices

None