HSP90 inhibition blocks ERBB3 and RET phosphorylation in myxoid/round cell liposarcoma and causes massive cell death in vitro and in vivo

Supplementary Materials

A gene expression meta-signature for myxoid liposarcoma.

The hypothesis addressed here is that if a meta-signature existed, the genes in the signature would reflect the essential transcriptional features of myxoid liposarcoma (MLA). There are 26 signatures analyzed for this case. At the significance threshold of Q-value less than 0.05, 87 genes were present in at least 22 of the 26 studies with minimum false discovery rate of 0.0714. All of the genes existing in this meta-signature are listed in Table S1.

We have used the limma package in R/Biocductor platform to calculate moderated t-statistics and p-values (1). Multiple testing issue was addressed by calculating q-values (2). To identify the meta-signature, we have modified and implemented the procedure described in (3) as follows:

1. A set S of differential expression analyses were selected and a significance threshold T was chosen to define differential expression signatures from the selected analyses (T_default = 0.10). Genes with a q-value below the threshold T were selected.
2. Genes were sorted by the number of signatures they were present in.
3. The number of genes present in each possible number of signatures was tallied (N_1, N_2, ..., N_26).
4. Random permutations were performed in which the actual q-values were assigned randomly to genes per signature, so that the genes in each signature changed randomly, but the number of genes in each signature remained the same. Randomization pattern was the same between signatures ensuring the dependence of genes across signatures during the randomization process. This simulation generated a tally of number of genes present in each possible number of random signatures (E_1, E_2, ..., E_26).
5. The significance of intersection among the true signatures was assessed by the minimum meta-false discovery rate (mFDRmin) as:
   \[ mFDR_{\text{min}} = \min\left(\frac{E_i + 1}{N_i}\right) \text{ for } i = 1 \text{ to } 26 \]
6. If mFDR_{\text{min}} was less than 0.10, a meta-signature was defined as those genes that were significantly differentially expressed (q ≤ T) in at least j of S analyses, where j was equal to i when mFDR_{\text{min}} was defined.
7. If no meta-signature was defined by using T_default steps 2 through 6 were repeated where T was lowered by 50% at each iteration until either a meta-signature was defined or the number of genes in two or more signatures reached zero.

The expression rate in Table S1 was defined as:
   \[ \text{Nummer om signature in which the is gene was significantly regulated} / \text{Number of signatures in which the gene was upregulated} \]
For example, AGT displayed an expression rate of 26/26, which means that AGT was upregulated in all signatures. GYG2 on the other hand showed an expression rate of 22/19, which means that the gene was differently expressed in 22 of 26 signatures. Nineteen of these 22 regulates were upregulations.

In the original method, the random signatures were generated without considering the dependency of genes across different comparisons. In our method, the same randomization pattern was applied to signatures that were generated from the same dataset, enabling a more realistic simulation. Another modification was to use moderated t-statistic instead of standard t-statistic when calculating p-values, thus enhancing the robustness of the test statistic.

Two public microarray datasets were analyzed to identify a meta-signature for MLS (Table S2). These datasets contained a range of sarcomas, including MLS, and we aimed to find a meta-signature of expressed genes for MLS by performing a two class differential expression analysis with MLS versus each of the different tumor types. In total, we have performed 26 comparisons, thus generated 26 signatures (Table S3).
# Table S1: A gene expression meta-signature for myxoid liposarcoma

| Gene symbol | Gene name                                                                                     | Expression rate |
|-------------|-----------------------------------------------------------------------------------------------|-----------------|
| AGT         | angiotensinogen (serpin peptidase inhibitor)                                                   | 26/26           |
| AKAP1       | A kinase (PRKA) anchor protein 1                                                               | 26/26           |
| CLK4        | CDC-like kinase 4                                                                              | 26/26           |
| CTAG2       | cancer/testis antigen 2                                                                       | 26/26           |
| EBF2        | early B-cell factor 2                                                                          | 26/26           |
| EMX2        | empty spiracles homeobox 2                                                                     | 26/26           |
| FAM13A1     | family with sequence similarity 13, member A                                                   | 26/26           |
| HOXA5       | homeobox A5                                                                                    | 26/26           |
| HSD11B2     | hydroxysteroid (11-beta) dehydrogenase 2                                                       | 26/26           |
| MYH15       | myosin, heavy chain 15                                                                        | 26/26           |
| OPRK1       | opioid receptor, kappa 1                                                                       | 26/26           |
| PTH2R       | parathyroid hormone 2 receptor                                                                 | 26/26           |
| PTX3        | pentraxin-related gene, rapidly induced by IL-1 beta                                            | 26/26           |
| RAB11FIP2   | RAB11 family interacting protein 2 (class I)                                                   | 26/26           |
| SHANK2      | SH3 and multiple ankyrin repeat domains 2                                                       | 26/26           |
| SIM1        | single-minded homolog 1 (Drosophila)                                                           | 26/26           |
| CA4         | carbonic anhydrase IV                                                                         | 25/25           |
| HOXA4       | homeobox A4                                                                                    | 25/25           |
| IDH1        | isocitrate dehydrogenase 1 (NADP +), soluble                                                  | 25/25           |
| KLFDC8A     | kelch domain containing 8A                                                                     | 25/25           |
| PPARG       | peroxisome proliferator-activated receptor gamma                                                | 25/25           |
| RET         | ret proto-oncogene                                                                             | 25/25           |
| SAMM50      | sorting and assembly machinery component 50                                                    | 25/25           |
| SCPEP1      | serine carboxypeptidase 1                                                                      | 25/25           |
| SH3PXD2A    | SH3 and PX domains 2A                                                                          | 25/25           |
| SLCO1C1     | solute carrier organic anion transporter 1C1                                                    | 25/25           |
| TAC1        | tachykinin, precursor 1                                                                        | 25/25           |
| CD36        | CD36 molecule (thrombospondin receptor)                                                        | 24/24           |
| CIB2        | calcium and integrin binding family member 2                                                    | 24/24           |
| FAM65B      | family with sequence similarity 65, member B                                                    | 24/24           |
| FZD4        | frizzled homolog 4 (Drosophila)                                                                | 24/24           |
| GPD1        | glycerol-3-phosphate dehydrogenase 1 (soluble)                                                 | 24/21           |
| KCNJ3       | potassium channel, subfamily J, member 3                                                        | 24/24           |
| MAN2A2      | mannosidase, alpha, class 2A, member 2                                                          | 24/24           |
| MAPK10      | mitogen-activated protein kinase 10                                                             | 24/24           |
| MOSC1       | MOCO sulphurase C-terminal domain containing 1                                                 | 24/21           |
| NMNAT2      | nicotinamide nucleotide adenylyltransferase 2                                                   | 24/24           |
| NRG2        | neuregulin 2                                                                                   | 24/24           |
| Gene   | Description                                      | Chromosome |
|--------|--------------------------------------------------|------------|
| PCNX   | pecanex homolog (Drosophila)                     | 24/24      |
| RERE   | arginine-glutamic acid dipeptide (RE) repeats    | 24/24      |
| SOX11  | SRY (sex determining region Y)-box 11            | 24/24      |
| ACACB  | acetyl-Coenzyme A carboxylase beta               | 23/23      |
| ACO1   |aconitase 1, soluble                              | 23/23      |
| ANGPT1 | angiopoietin 1                                   | 23/23      |
| CIorf115| chromosome 1 open reading frame 115              | 23/23      |
| CITED1 | Cbp/p300-interacting transactivator 1            | 23/23      |
| CTAG1B | cancer/testis antigen 1B                         | 23/23      |
| FGFR2  | fibroblast growth factor receptor 2              | 23/23      |
| FMO2   | flavin containing monooxygenase 2 (non-functional)| 23/23      |
| GNAT3  | guanine nucleotide binding protein, alpha transducing 3 | 23/23 |
| HSDL2  | hydroxysteroid dehydrogenase like 2              | 23/23      |
| ITIH5  | inter-alpha (globulin) inhibitor H5              | 23/23      |
| LIPE   | lipase, hormone-sensitive                        | 23/20      |
| PGRMC2 | progesterone receptor membrane component 2       | 23/23      |
| PLIN   | perilipin                                        | 23/19      |
| PTGER3 | prostaglandin E receptor 3 (subtype EP3)         | 23/22      |
| RPL31  | ribosomal protein L31                            | 23/23      |
| SEMA3G | sema domain, short basic domain (semaphorin) 3G  | 23/20      |
| TMEM135 | transmembrane protein 135                       | 23/23      |
| ACAA1  | acetyl-Coenzyme A acyltransferase 1              | 22/22      |
| ADIPOQ | adiponectin, C1Q and collagen domain containing  | 22/22      |
| ALDH1L1| aldehyde dehydrogenase 1 family, member L1      | 22/22      |
| AQP7   | aquaporin 7                                      | 22/19      |
| BBOX1  | (gamma-butyrobetaine hydroxylase) 1              | 22/22      |
| CAMK1  | calcium/calmodulin-dependent protein kinase I    | 22/22      |
| CSAD   | cysteine sulfinic acid decarboxylase             | 22/22      |
| DHDDS  | dehydrodolichyl diphosphate synthase             | 22/22      |
| DTX4   | deltex homolog 4 (Drosophila)                    | 22/22      |
| ECHDC3 | enoyl Coenzyme A hydratase domain containing 3   | 22/22      |
| EHBP1  | EH domain binding protein 1                      | 22/21      |
| EPB41L4B| erythrocyte membrane protein band 4.1 like 4B   | 22/22      |
| FABP4  | fatty acid binding protein 4, adipocyte          | 22/22      |
| GCSH   | glycine cleavage system protein H (aminomethyl carrier) | 22/22 |
| GYG2   | glycogenin 2                                     | 22/19      |
| HOXA9  | homeobox A9                                      | 22/22      |
| HPGD   | hydroxyprostaglandin dehydrogenase 15-(NAD)     | 22/22      |
| ISOC1  | isochorismatase domain containing 1              | 22/22      |
| Gene   | Description                                                                 | Coverage |
|--------|-----------------------------------------------------------------------------|----------|
| LEPR   | leptin receptor                                                             | 22/22    |
| LEPREL1| LEPREL1 collagen prolyl hydroxylase                                         | 22/22    |
| LOC730107| similar to Glycine cleavage H protein, mitochondrial                      | 22/22    |
| LPL    | lipoprotein lipase                                                          | 22/22    |
| PEG3   | paternally expressed 3                                                     | 22/22    |
| PPFIBP2| PTPRF interacting protein, (liprin beta 2)                                 | 22/22    |
| RPL23AP13| ribosomal protein L23a pseudogene 13                                       | 22/22    |
| RREB1  | ras responsive element binding protein 1                                   | 22/22    |
| SPINK5 | serine peptidase inhibitor, Kazal type 5                                   | 22/22    |
| UNG    | uracil-DNA glycosylase                                                      | 22/22    |

**Table S2: The microarray datasets that were analyzed to generate a meta-signature**

| Study     | Authors       | PMID         | Array type | Number of samples |
|-----------|---------------|--------------|------------|-------------------|
| GSE6481   | Nakayama et al. | 17464315    | Hgu133a    | 105               |
| E-MEXP-353| Hendersen et al.| 16168083    | Hgu133a    | 96                |

**Table S3: Differential expression classes**

| Study     | Class 1 (# of samples)                                                                 | Class 2 (# of samples)                                                                 |
|-----------|----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| GSE6481   | MLS (7)                                                                                 | Osteosarcoma (11)                                                                      |
|           |                                                                                        | Alveolar Rhabdomyosarcoma (4)                                                          |
|           |                                                                                        | Chondroblastoma (4)                                                                    |
|           |                                                                                        | Chondromyxoid Fibroma (4)                                                              |
|           |                                                                                        | Chondrosarcoma (7)                                                                    |
|           |                                                                                        | Chordoma (4)                                                                           |
|           |                                                                                        | Dedifferentiated Chondrosarcoma (3)                                                    |
|           |                                                                                        | Embryonal Rhabdomyosarcoma (3)                                                         |
|           |                                                                                        | Fibromatosis (5)                                                                      |
|           |                                                                                        | Leiomyosarcoma (8)                                                                    |
|           |                                                                                        | Lipoma (3)                                                                             |
|           |                                                                                        | Malignant Peripheral Nerve Sheath Tumor (4)                                            |
|           |                                                                                        | Monophasic Synovial Sarcoma (10)                                                       |
|           |                                                                                        | Neurofibroma (4)                                                                       |
|           |                                                                                        | Sarcoma (3)                                                                            |
|           |                                                                                        | Schwannoma (4)                                                                         |
|           |                                                                                        | Well-differentiated Liposarcoma (3)                                                     |
| E-MEXP-353| MLS(19)                                                                                  | Dedifferentiated liposarcoma (15)                                                      |
|           |                                                                                        | Fibrosarcoma (4)                                                                       |
|           |                                                                                        | Leiomyosarcoma (6)                                                                     |
|           |                                                                                        | Lipoma (3)                                                                             |
|           |                                                                                        | Malignant.fibrous.histioctyoma (21)                                                     |
|           |                                                                                        | Malignant périphéral.nerve.sheath.tumor (3)                                            |
|           |                                                                                        | Myxofibrosarcoma (15)                                                                 |
|           |                                                                                        | Synovial.sarcoma (16)                                                                  |
|           |                                                                                        | Well-differentiated.liposarcoma (3)                                                     |
Supplementary Figure S1: Growth/survival MLS cell lines titration Vandetanib. 

(A) Growth/survival assay of indicated cell lines with up to 50,000 nM of Vandetanib. 

(B) RET phosphorylation in MLS cell lines treated with Vandetanib. Western blot analysis of RET Y905 phosphorylation performed in whole cell lysates as described in Olofsson et al 2004. Vandetanib concentrations in nM.

(C) Growth/survival assay of indicated cell lines with up to 1,00,000 nM Gefitinib.

(D) Growth/survival assay with up to 20,000 nM of Vandetinib in presence of 25,000 nM of Gefitinib.
Supplementary Figure S2: Eosin-hematoxylin stained sections of xenografted MLS tumor tissues. Samples from control mouse and 17-DMAG treated animals as indicated. Pictures captured with a 20× objective and a ProGres® digital microscope camera.
Supplementary Film Clips: 3D photographs of PLA signals (red dots) from single ERBB3, RET and combined ERBB3 and RET antibodies in MLS 402.91 cells. Original photos were captured using a Zeiss 700 confocal system. DNA (nuclei) were stained with DAPI (Blue). Film clips were created with the Volocity software. The DAPI signal was faded in order to show the intranuclear PLA signals.

REFERENCES

1. Smyth GK. limma: Linear Models for Microarray Data. In: Gentleman R, Carey VJ, Huber W, Irizarry RA, Dudoit S, editors. Bioinformatics and Computational Biology Solutions Using R and Bioconductor. Springer New York. 2005; 397–420.

2. Storey JD. A direct approach to false discovery rates. J R Stat Soc Ser B Stat Methodol. 2002; 64:479–98.

3. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, et al. Large-scale meta-analysis of cancer microarray data identifies common transcriptional profiles of neoplastic transformation and progression. Proc Natl Acad Sci USA. 2004; 101:9309–14.