

Overview

Tandem mass tag (TMT) sixplex [1] labelling was applied to develop a panel of protein biomarkers that could be useful for testing the allergic potency of new chemicals in cell-based bioassays.

Introduction

Common environmental and industrial small molecular weight chemicals can cause allergic contact dermatitis (ACD).

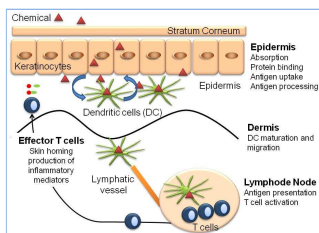


Fig. 1: Key events in the skin and lymph nodes upon exposure to a sensitizing chemical

Current skin sensitization testing predominantly relies upon the use of animal test methods. With the forthcoming ban on animal testing for cosmetic ingredients in the European Union there is a great need for alternative in vitro tests. The aim of the European Framework 6 funded Sens-it-iv project is to develop "in vitro" alternatives to animal tests [2].

Methods

Chemicals
Human primary keratinocytes from four donors were incubated for 24 h with a set of training chemicals comprising five skin sensitizer and two non-sensitizer/irritant at different non-toxic concentrations (n=8 per treatment, Table 1). Cell viability was measured by LDH assay (>75%).

Chemical	Structure	Potency	Concentration
cinnamic aldehyde (Cin-A)		moderate	5 / 18 µg/ml
2,4-dinitrochlorobenzene (DNCB)		strong	4 / 8 µM
2,4-dinitrobenzene sulphonic acid (DNBS)		strong	4 / 8 µM
tetramethyl thiram disulfid (TMTD)		moderate	2 / 3,2 µg/ml
NI _{SO₄}		non-sensitizer	0.25 / 5 mM
sodium lauryl sulphate (SLS)		non-sensitizer	14 / 17 µg/ml

Table 1: Classification of test compounds

TMT Labelling and LC-MS/MS

After cell lysis, proteins were digested with trypsin, labelled with TMTsixplex (Pierce Biotechnology) using a randomized block design in which samples of one treatment group were blocked but randomized with regard to TMTsixplex (Fig. 2).

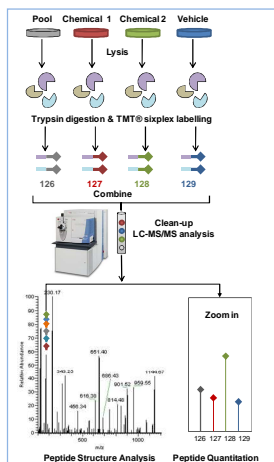


Fig. 2: TMT@ Quantification workflow

A reference pool was created from an aliquot of all samples and included in each TMTsixplex LC-MS/MS measurement. All spectra were acquired on a Thermo LTQ-Orbitrap XL operated in CID and HCD mode. The peptide mixture was separated on an Eksigent NanoLC using a Reprosil C18 trapping column (10 x 0.1 mm, 5 µm) and a Reprosil C18 analytical column (400 x 0.075 mm, 3 µm) at a flow rate of 350 nL/min (60 min gradient: 5-30% acetonitril). Instrument parameter: Resolution (MS = 30,000; MS/MS = 7,500), collision energy (CID = 3.5 and HCD = 75) isolation width (CID=2 u, HCD=1.5 u).

Data Analysis and Statistics

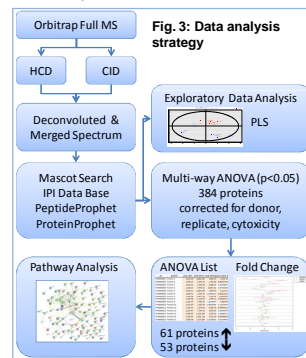


Fig. 3 shows the data analysis workflow. Correction of reporter ion intensities was performed to adjust for isotopic impurities and systematic bias by means of sum scaling. Protein abundance values were computed as normalized log₂ values of reporter ion to reference ratios.

Statistical analysis was performed using the statistical scripting language R or the data analysis software MeV (TIGR) version 4.3. Statistically significant regulated proteins between the experimental groups (control,

sensitizer, irritant) were identified using multi-way ANOVA (p<0.05) corrected for donor, replicate and cytotoxicity. ANOVA uses those proteins that have valid measurements (384 in total). To determine whether the intensity levels of these proteins could discriminate between the experimental groups we used an unsupervised method, partial least square regression (PLS) analysis (software SIMCA-P 11.0) [3].

Results

Data Structure

To identify biomarkers showing consistent regulation among the experimental groups a total of 168 samples from four donors were analyzed.

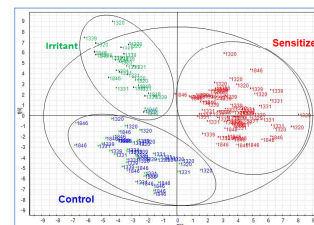


Fig. 4: PLS score plot component 5 and 6

Trends and variability in the data set were analyzed by PLS analysis. For most of the proteins the biggest source of variation was donor-to-donor variation (i.e. biological). Fig. 4 shows a PLS score plot of the data set (Y-response vector: chemical, donor, replicate, cytotoxicity; X-vector: referenced protein abundance values). Each label represents a particular sample, and is automatically color-coded according to its class. Components 5 and 6 show a good separation between the experimental groups. To determine which proteins are important to separate the groups a PLS loading plot was generated. Fig. 5 shows a loading plot which describes the weighting coefficients for each protein.

Biomarker discovery

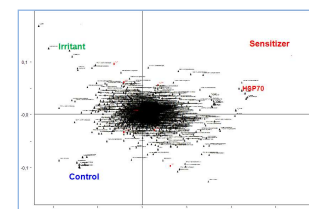


Fig. 5: PLS loading plot component 5 and 6

Analysis of variance (ANOVA) was used to test for differences between the experimental groups and included covariates to control for cytotoxic or donor effects. In total, 113 proteins were selected by the ANOVA analysis (p<0.05). Fig. 6 shows the log₂-transformed and referenced fold changes of 15 protein biomarkers.

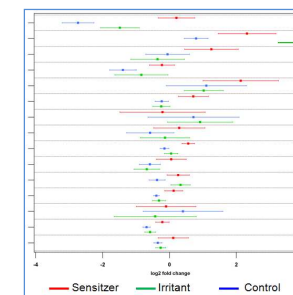


Fig. 6: Log 2 fold changes of biomarkers

Among the proteins showing differential level between sensitizer and irritant treated samples HSP70 was chosen for further verification. Fig. 7 shows a representative experiment that illustrates TMT (A) and ELISA-based (B) quantitation of heat shock protein 70 (HSP70) in one donor. HSP70 levels were increased in CA and TMTD

treated samples. HSPs are constitutively expressed in the skin and can be up-regulated as a result of exposure to stresses. Inhibition of HSP70 in a murine model of allergic contact hypersensitivity led to the induction of tolerance to 1-fluoro-2,2-dinitrobenzene (DNFB) [4].

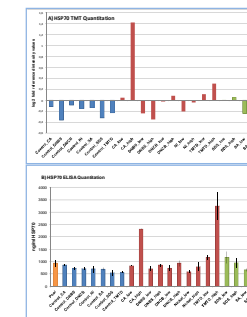


Fig. 7: HSP70 levels in one donor

Conclusions

A top list of 113 protein biomarkers was identified that can be used to differentiate between sensitizer, irritant or control treated samples. HSP70 was verified by ELISA and responded to a subset of chemicals, only. Only proteins most strongly associated with sensitizer exposure will be taken forward in a more focused quantitative MS assay.

References

- Dayon L et al. (2008). Anal Chem. 80(8):2921-31.
- Weltzien HU et al. (2009). Journal für Verbraucherschutz und Lebensmittelsicherheit 4: 41-48
- Palermo G et al. (2009). Computational Biology and Chemistry: Advances and Applications 2: 57-70
- Yusuf N et al. (2009). J Immunol. 182(1):675-83.

Acknowledgements

The authors gratefully acknowledge the funding from the EU 6th Framework Sens-it-iv Project (novel testing strategies for in vitro assessment of allergens, LSHB -CT-2005-018681) (<http://www.sens-it-iv.eu>).