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BIOENGINEERED TATTOO; A PROMISING BIOMARKER FOR THE EARLY DETECTION OF CANCER: A REVIEW

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ABSTRACT

Early detection increases the chance of survival and is essential for a successful therapy. Numerous types of cancer are diagnosed late, which diminishes the efficiency of treatment and the outlook is less promising. Some cancers show increased level of calcium in bloodwhereas, some type of cancers shows non – specific symptoms including anorexia, anxiety and weakness. Based on this concept, scientists developed a tattoo using engineered cells which acts as a biomarker for the early detection of cancer. These cells contain specific calcium sensing receptors (CaSR) that activates transgenic tyrosinase enzyme, which produces melanin in response to persistently elevated blood calcium. This melanin generated tattoo could be detected with the naked eye. CaSR is a natural calcium sensing receptor which detects mild to moderate hypercalcemia (5.6 – 10 mcg ca^{2+}/dL blood). This system is validated in wild type mice bearing engineered cells, injected with hypercalcemic breast and colon adenocarcinoma cells and develops tattoo. No tattoo were developed in any animal injected with normocalcemic tumor cells. The tattoo is recognizable for four of the major widespread types of cancers – which are often detected late – namely: breast cancer, lung cancer, prostate cancer, colon cancer gastrointestinal and hematological malignancies. However hypercalcemia may also occur due to any metabolic changes or pathological conditions. Persistent hypercalcemiac condition develops large tattoo on skin surface implanted with these engineered cells. This is the distinguishing feature from normal hypercalcemic and may regarded as the early detection of cancer.

INTRODUCTION

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. These contrast with benign tumours, which do not spread. Early detection of cancer greatly increases the chances for successful treatment. There are two major components of early detection of cancer:a) Education to promote early diagnosis and screening. b) Recognizing possible warning signs of cancer and taking prompt action leads to early diagnosis.

Some early signs of cancer include lumps, sores that fail to heal, abnormal bleeding, persistent indigestion and weight loss, changes in bowel movements and chronic hoarseness. Early diagnosis is particularly relevant for cancers of the breast, cervix, mouth, larynx, colon and rectum, and skin.Due to the rapid technological advances of the last years, it is now possible to analyse the molecular makeup of different cancer types in detail within short time periods. At the earliest stages of cancer

development, blood levels of calcium become super-elevated in a phenomenon known as "hypercalcemia".[1] Studies have reported that 30 percent of individuals diagnosed with a form of cancer have an elevated calcium concentration in their systems.This concept helps the scientist to design a biomedical tattoo that changes the color when calcium in blood is too high.When calcium levels are higher than normal, the cells produce melanin, a dark pigment that's already found in body. This increased calcium levels are detected by specific calcium sensing receptors (CaSR) which are implanted under the skin. This appear as large mole which can be detected with the naked eye. As a natural skin pigment, melanin is particularly well suited for a biomedical tattoo. The tattoo was insensitive to short-term Ca^{2+} fluctuations as well as leaky accumulation and was stable for at least 6 months.[1] Experiments have been conducted in wild type mice which are injected with hypercalcemic tumor cells and shows visible tattoo whereas, no tattoo were observed in animals injected with normocalcemic tumor cells under 38 day experimental period.

ROLE OF CALCIUM IN HUMAN BODY

Calcium plays a major role in normal homeostatic mechanism. Calcium acts as a second messengervia rapid, as well as more sustained changes in intracellular calcium levels through the actions of calcium channels, exchangers and pumps. However calcium also acts as a first messenger through a G-protein coupled receptor that senses extracellular calcium. It is involved in regulating various cellular functions like cell proliferation, cell differentiation, cell adhesion and motility as well as apoptosis. Calcium is stored in bones as mineralized form and present in blood in free form as well as inbound form with plasma proteins. The calcium homeostasis in human body is regulated by Parathyroid Hormone (PTH) and Calcium sensing receptors (CaR). Increased level of calcium in blood is restored within minutes by normal body's homeostatic mechanisms.[2] Blood calcium levels are regulated by parathyroid hormone (PTH), which is produced by the parathyroid glands. PTH is released in response to low blood calcium levels. It increases calcium levels by targeting the skeleton, the kidneys, and the intestine. In the skeleton, PTH stimulates osteoclasts, which are cells that cause bone to be reabsorbed, releasing calcium from bone into the blood. PTH also inhibits osteoblasts, cells which deposit bone, reducing calcium deposition in bone. In the intestines, PTH increases dietary calcium absorption and in the kidneys, PTH stimulates re-absorption of the calcium. While PTH acts directly on the kidneys to increase calcium re-absorption, its effects on the intestine are indirect. PTH triggers the formation of calcitriol, an active form of vitamin D, which acts on the intestines to increase absorption of dietary calcium. PTH release is inhibited by rising blood calcium levels.[2]

CACIUM SENSING RECEPTORS (CaSR)

The CaSR is important in maintaining and regulating mineral ion homeostasis. However, the receptor is also widely expressed in tissues not involved in calcium homeostasis and modulates various cellular functions, including gene expression, proliferation, differentiation, apoptosis and chemotaxis. CaSR has the ability to function as a sensitive detector of changes in calcium concentrations. Stimulation of the receptor can be converted into activation of many signaling pathways known to be used by the CaSR. CaSR mostly expresses in parathyroid cells and human embryonic kidney (HEK-293) cells.

CaSR activates MAPKs (Mitrogen activated protein kinase). Activation of MAPKs has been shown to be important for many distal effects of CaSR such as proliferation, differentiation, regulation of peptide and ion channel activity. CaSR is believed to desensitize very slowly, beta-arrestins and GKs seem to mediate CaSR desensitization in vitro. In addition, this study elegantly showed that beta-arrestins 2- null mice seen to have leftward shift in calcium parathyroid hormone relationship. Beta-arrestins are also important for regulating CaSR function in parathyroid gland in vivo. CaSR in the parathyroid gland has a central role in calcium homeostasis. A reduction in calcium plasma concentration results in CaSR mediated increase in PTH secretion from the parathyroid cells. The increased PTH level promotes distal renal tubule calcium reabsorption and bone

reabsorption by the lining cells, both of which increase calcium levels. Furthermore the relative hypocalcaemia also leads to reduced secretion of calcitonin from thyroid C cells mediated by CaSR, preventing inhibition of bone resorption by calcitonin. Both PTH and a low calcium level induce synthesis of Vitamin D_3 in the proximal tubular cells of kidney. The vitamin D_3 metabolite stimulates intestinal calcium absorption. The reverse of these events occurs in hypercalcemia.[3,4]

HYPERCALCEMIA AND CANCER

Hypercalcemia is a common complication of malignant disease that is of clinical importance because of its association with symptomatic deterioration and death. Hypercalcemia is an important cause of morbidity and mortality in patients with cancer. Hypercalcemia is a marker of advanced cancer rather than the actual cause of death in many cases. Although follow-up data were admittedly incomplete, uncontrolled hypercalcemia appeared to contribute directly to death in only 11 of the 40 patients (28%) in whom follow-up serum calcium levels were measured. certain patients with biochemical evidence of humoralhypercalcemia of malignancy responded slightly less well to osteoclast inhibitors than those with local osteolytichypercalcemia because of the effects of parathyroid-hormone-related peptides on the renal tubule. There was little difference between symptomatic response in patients with tumors where one would have expected a mainly humoral mechanism of hypercalcemia to be operative (squamous carcinomas) and those in whom local osteolytic mechanisms of hypercalcemia may have been more common (breast and hematologic tumors).[5,6] Hypercalcemia is associated with most widespread types of cancer namely: breast cancer, lung cancer, colon cancer and prostate cancer. Primary tumor cells metastases and attacks the immune cells that act on bone, kidney, and intestine which interrupts calcium homeostasis which increases blood calcium concentration. The researchers utilized the calcium sensing receptor (CaSR) which naturally functions with high sensitivity to increased levels of Ca^{2+} in the blood. Increased Ca^{2+} concentration activate CaSR to inhibit the secretion of parathyroid hormone. CaSR is capable of detecting mild to moderate blood hypercalcemia, as defined by the study as 5.6 to 10mg/dL, which often goes unnoticed in the patient during very early stages of cancer growth. The biomedical tattoo, made up of engineered cells to express tyrosinase, have a rewired signaling pathway for CaSR activation. Upon CaSR activation, the redesigned signaling cascade leads to expression of tyrosinase which produces melanin. The design was intended to detect the accumulation of melanin as a pigmented mark, or tattoo that could signal the long-term presence of blood hypercalcemia. The body's normal Ca^{2+} homeostasis mechanisms would not be affected because the feedback pathways between blood Ca^{2+} and PTH secretion respond within minutes of an increase or decrease in Ca^{2+} concentration.[7]

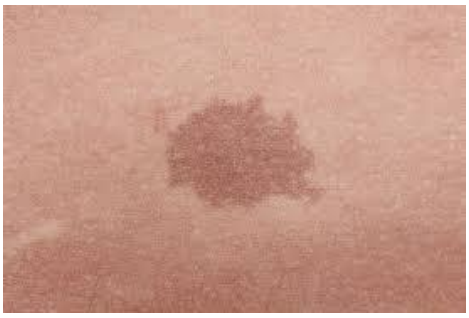

Fig No 1: subcutaneously implanted HEK_{Tattoo} cells produces melanin on account of hypercalcemia

MELANIN: THE NATURAL BIOMARKER[8]

Melanin is natural pigment present in human body. Due to its characteristic color scientists had developed melanin based tattoo. The enzyme tyrosinase converts tyrosine to DOPA which undergo serious transformations to produce a dark colored pigment known as melanin. Stimuli of melanogenesis include sunlight or UV radiations, androgens, which is a natural steroid hormone, and inflammatory mediators. Melanin synthesis takes place in specialized cells called melanocytes within membrane bound organelles called melanosomes.

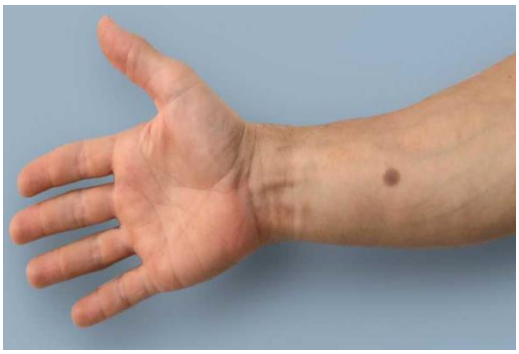

Fig No 2: Formation of Tattoo by HEK_{Tattoo} cells

This tattoo focuses on the activation of tyrosinase enzyme which increases melanin production on increased blood calcium level associated with cancer. The use of a biomedical tattoo would be best indicated for:

a) Individuals with known risk factors for colon cancer and breast cancer.

b) For patients who have undergone primary tumor treatmentand to monitor the recurrence of cancer.

c) Individuals who have a previous family history in developing cancers.

This article mainly deals with the design, construction and validation of biomedical tattoo in wild type mice in vitro. The idea would still need to be studied further in animals and in patients before it could ever be used in the clinic. "Potentially, such a tattoo could be linked to something that is slowly developing, like a neurodegenerative disorder such as Parkinson's."[9]

PROPERTIES

- It detectspersistant increased calcium concentration in the blood by producing a black mole.It is insensitive to short-term calcium fluctuations in the body.It can be detected with the naked eye.
- It is stable for 6 months.It does not affect the body's normal calcium homeostasis mechanism.
- Microencapsulation is achieved to prevent host immune cell attack.

ADVANTAGES

- It is intended primarily for self-monitoring, making it very cost effective.
- It is also effective to detect any neurodegenerative diseases or hormonal disorders in the early phases. E.g. Parkinson's disease.

DISADVANTAGE

- The appearance of mole may sometimes create a fear of death in the patient.It does not have a long "shelf life," so it would have to be "updated" repeatedly.
- Encapsulated living cells last for about a year, after that, they must be inactivated and replaced.
- Another catch is that this implant is, as yet, only an early prototype, and much more research is needed before it can be put to the test on humans. The road to making the biomedical tattoo available for use is long and laborious.
- Continued development and clinical trials in particular are laborious and expensive.

WORKING OF THE BIOMEDICAL TATTOO[10]

The cell engineered tattoo consists of two main components:A hypercalcemic sensor that monitors increased blood Ca2+, A promoter for the expression of tyrosinase enzyme. Hypercalcemia activates CaSR which triggers NFAT (Nuclear Factor of Activated T cells) and SRE (Serum Response Element).This induces the synthetic calcium sensitive promoter (P_{ca}) in engineered cells which expresses human tyrosinase enzyme, the rate limiting enzyme in melanin synthesis.

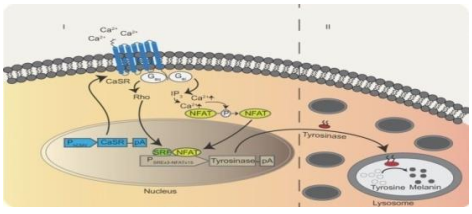

Fig No 3: Molecular mechanism of pigment production by HEK_{Tattoo} cells upon induction of hypercalcaemia

Thus, on account of hypercalcemia, subcutaneously implanted engineered cells produce visualized black skin tattoo due to accumulation ofmelanin.

DESIGN OF STABLE HEK$_{TATTOO}$

- The HEK-293 cells are the suitable cell lines for detection of hypercalcemia.
- The tyrosinase expression vector is transfected with HEK-293 cell lines for efficient tyrosinase activity and optimization of melanin production.[11]
- The tyrosinase activity exceeds in HEK-293 cells than all other cell lines.
- Quantitative analysis of pigment production by HEK $_{Tattoo}$ under mild and moderate hypercalcemic conditions indicates that only persistent hypercalcemia for 12 hours (mild hypercalcemia) and 24 hours (moderate hypercalcemia) triggers melanin production.[12,13]
- The engineered biomedical tattoo is insensitive to normal calcium fluctuations because the calcium homeostasis is retained within minutes. This is due to the interplay between calcitonin & PTH.
- To evaluate the **dynamics of melanin production**, the HEK $_{Tattoo}$ cells are seeded with increasing concentration of calcium.

➢ Low Ca^{2+} conc. (0.5mM) – No pigment production.

➢ Normal Ca^{2+} conc. (1.3mM) – No pigment production.

➢ Mild hypercalcemia (1.6mM) – Pigment production.

➢ Moderate hypercalcemia (1.8mM) – Pigment production.

- The **stability of HEK –Tattoo** were determined by storing hypercalcemia induced melanin containing HEK-Tattoo pellets at room temperature for 6 months. The dark pigment remains stable for the entire period.[14]

DETECTION OF CANCER INDUCED HYPERCALCEMIA IN NUDE MICE

Hypercalcemia induced melanin production by HEK$_{Tattoo}$ via a visible skin tattoo was detected by injecting nonencapsulatedHEK$_{Tattoo}$ cells into the flank of nude mice with mammary as well as colon adenocarcinoma cells or normocalcemic adenocarcinoma cells. The hypercalcemic adenocarcinoma cells were produced by inoculation of 410.4 or colon 26 cells. Similarly the normocalcemic adenocarcinoma cells were produced by inoculating 168 cell line; the negative control. Non encapsulated HEK-293 cells engineered for tyrosinase expression (pAT10, P_{hCMV} –Tyr –pA) injected into healthy nude mice and were used as a positive control.[15]

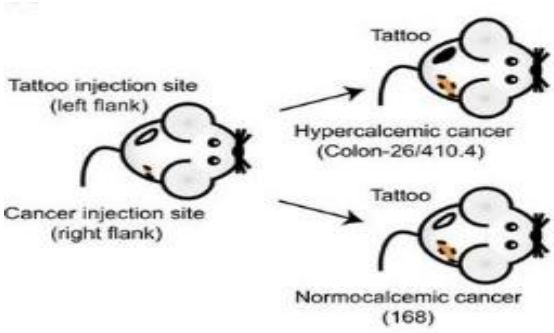

Fig No 4: Schematic representation of nonencapsulatedHEK$_{Tattoo}$ cells in nude mice injected with hypercalcemic and normocalcemic tumor cells

In vivo studies have shown that, hypercalcemia induced melanin production by HEK$_{Tattoo}$ via a visible skin tattoo was detected by injecting nonencapsulatedHEK$_{Tattoo}$ cells into the flank of nude mice with mammary as well as colon adenocarcinoma cells and normocalcemic adenocarcinoma cells. For better stability, the implants were microencapsulated with alginate-PLL-alginate beads.[16]

VALIDATION OFHEK$_{Tattoo}$IN WILD - TYPE MICE

Microencapsulated HEK$_{Tattoo}$were subcutaneously implanted into the flank of wild- type mice with hypercalcemic or normocalcemic adenocarcinoma cells. Hypercalcemic adenocarcinoma cells were produced by the inoculation of 410.4 or colon-

26 cells. Normocalcemic adenocarcinoma cells were produced by the inoculation of 168 cell line; negative control.[17]

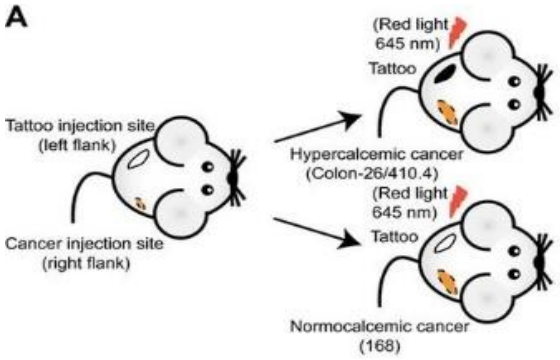

Fig No 5: Schematic representation of microencapsulated engineered HEK$_{Tattoo}$ cells implanted into wild-type mice with hypercalcemic and normocalcemic tumor cells

Microencapsulated HEK -293 cells engineered for tyrosinase expression (pAT10, P_{hCMV} –Tyr-pA) were injected into healthy mice which serves as a positive control. Animals injected with normocalcemic cancer cells serves as negative control, do not produce any visible tattoo as expected. But animals injected with hypercalcemic cancer cells produces a visible tattoo throughout the 38 day experimental period.[17]

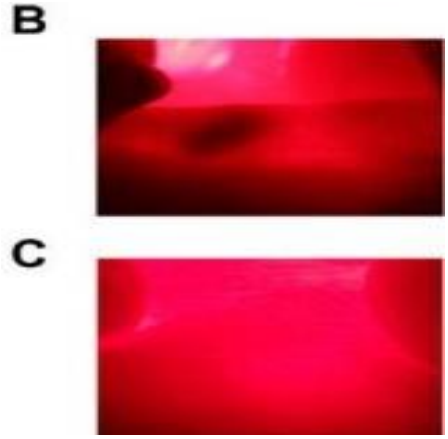

Fig No: 6Image B and C indicate the photograph of positive and negative control mice illuminated with red light respectively.

CONCLUSION

The aim of the biomedical tattoo is to detect asymptomatic mild hypercalcemia associated with cancer. Hypercalcemia develops from mild via moderate to high hypercalcemia. The biomedical tattoo detects persistent mild hypercalcemia, which may indicate an emerging health issue that requires further attention. In mouse models inoculated with colon and breast cancer cell lines known to be associated with hypercalcemia, the biomedical tattoo reliably detected the development of cancer that was still at an asymptomatic stage. Such a system would have practical benefits, because diagnosis of asymptomatic cancer, although difficult, could increase treatment options and improve survival rates, particularly for individuals with known risk factors and patients who have undergone primary tumor treatment and require continuous monitoring to diagnose cancer recurrence or development of metastases. Therefore, the use of a biomedical tattoo for the diagnosis of hypercalcemia associated with cancer would be best indicated for individuals with known risk factors for colon cancer [loss of CaSR expression, ectopic PTH secretion, and ulcerative colitis] and breast cancer [human epidermal growth factor receptor-2–positive status, parathyroid hormone-related protein overexpression, and ectopic expression of PTH] and for patients who have undergone primary tumor treatment and require continuous monitoring to diagnose cancer recurrence, as well as the development of metastases. In conclusion, the present work provides an important proof of concept of this new diagnostic strategy, demonstrating the feasibility of using engineered cell–based biomedical tattoos for surveillance and potential detection of asymptomatic cancers based on mild hypercalcemia.

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