3. MATERIALS AND METHODS

3.1. Animals and maintenance.

NOD SCID mice of 6-8 weeks old age were procured from the Animal Facility of the ACTREC, Navi Mumbai. The proposal for use of NOD SCID mice was sanctioned by the Institutional Animal Ethics Committee (IAEC) of the ACTREC (Annexure 1) which is duly endorsed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India. The animals were housed in groups of 4-5 animals per polysulfone make individually ventilated cage (Citizen Industries, Ahmedabad, India) under controlled conditions of 55 ± 5% humidity, 24 ± 2°C temperature, and 12-h light/12-h dark cycle under specific pathogen-free conditions. All mice were maintained on sterilized corn-cob bedding material. All mice were handled for experimentation in a cage changing station (Labconco, Kansas, USA) with sterile technique. All mice were maintained on in-house pelleted animal feed and UV treated water ad libitum.

3.2. Collection of fresh surgical human tumor samples from the operation theater.

The Biorepository Laboratory of ACTREC has collected the surgical tumor samples from the operation theater of ACTREC after obtaining due informed consent of the patients undergoing surgeries. These tumor samples were requested to be collected in the RPMI 1640 medium (Invitrogen, USA) containing antibiotics. Viable tumor tissues of the available tumor type of brain, breast, head and neck (oral cavity) and bone and soft tissues (muscle) were collected from the Biorepository Laboratory of ACTREC in 15 ml capacity sterile tubes (Tarsons, Kolkata, India) and were brought on ice to the Animal Facility and were implanted in the animals within an hour of collection from the patients.

3.3. Fresh human tumor implantation in NOD SCID mice.

Connective, necrotic or suspected necrotic tissue as well as blood clots, if any, was removed by use of sharp scalpel blade. Tumor tissue were cut in small pieces of 5x5 mm blocks and then rinsed three times using RPMI 1640 medium. NOD SCID mice were anaesthetized by combination of 2% isoflurane gas (Abbott Laboratories Ltd., Kent, UK) and 100% oxygen at 3 lit/min to achieve the surgical anesthesia by
using the isoflurane gas anesthesia assembly (VetEquip, Inc., Pleasanton, USA). Hairs were clipped by use of hair clipper (Roboz Surgicals, USA) and skin was wiped with the help of 70% alcohol. For brain, head and neck (oral cavity) and bone and soft tissues (muscle) tumors, small skin incision of 3-4 mm size was made with the help of pointed scissor in the back region at lumbar vertebra level. Small pocket was created with the help of pointed scissor under the skin at the level of paralumbar fossa by blunt dissection. Tumor pieces of brain, head and neck (oral cavity) and bone and soft tissues (muscle) were slipped from the opening under the skin and retained below the paralumbar fossa as per the standard protocol (Furukawa et al., 1993; Teraoka et al., 1995; Morton and Houghton, 2007 and Frapolli et al., 2010). Skin wound was sealed with the help of surgical glue, Vetbond (3M, USA). For brain tumor tissues, 18 different primary samples were implanted in 37 mice. For oral cavity tumor tissues, 13 different primary samples were implanted in 28 mice. For muscle tumor tissues, 5 different primary samples were implanted in 16 mice.

For breast tumor tissues, small skin incision was made at the paralumbar fossa level and small pocket was made near the mammary fat pad in a similar fashion as above. Breast tumor pieces were slipped from the opening in the skin and retained orthotopically in the vicinity of the mammary tissue. Skin wound was sealed with the help of surgical glue, Vetbond (3M, USA). For breast tumor tissues, 14 different primary samples were implanted in 38 mice.

3.4. Criteria for animal sacrifice, tumor measurement and end point.

Tumor growth was measured with the help of vernier caliper [Mitsuyoto, Kawasaki, Japan] (Ewens et al., 2005). After the tumor transplant, NOD SCID mice were maintained atleast till the tumor grew to 15 mm size in diameter or maximum up to 9 months from the date of tumor transplant for the metastasis observation. Time taken to achieve 15 mm tumor size in SCID mice was recorded. Some of the animals were maintained till the tumor grew to 30-35 mm to see metastasis, if any.

3.5. Animal sacrifice and sample collection.

After the tumor was grown in these mice, animals were sacrificed by CO₂ inhalation and tumor was excised aseptically. Small tumor pieces were frozen down in LN₂ using 2 ml capacity screw cap vials containing 40% RPMI 1640, 50 % FBS (Invitrogen, USA) and 10 % DMSO (Sigma Chemicals, USA) as per the standard
protocol (Gill, 2010). Small piece of tumor as well as suspected areas of lung and liver tissues were snap frozen in LN2 container (Biogenics, California, USA, model-MVE XC47/11-6) using 2 ml capacity screw cap sterile SV2 vials (Tarsons, Kolkata, India) and stored in -80 refrigerator (New Brunswick Scientific, England; model-U570 Premium) for DNA extraction to be used for ascertaining human origin of the tumors as well as metastasis using STR method. Small piece of tumor tissue and vital organs like lungs, liver, kidney, spleen and brain were fixed in buffered neutral formalin, fixed at least for 24 h and were processed for histopathology studies. Typically five microns thickness of sections of the paraffin embedded tissues was cut and stained using standard H & E staining (Coolidge and Howard, 1979).

3.6. Serial transplantation of the tumor in fresh animals.

Tumors grown in above mice were transplanted by serial transplantation in few fresh SCID mice as above. In short, connective, necrotic or suspected necrotic tissue as well as blood clots, if any, was removed by use of sharp scalpel blade. Tumor tissue were cut in small pieces of 5x5 mm blocks and then rinsed three times using RPMI 1640 medium. NOD SCID mice were anaesthetized by 2% isoflurane gas to achieve the surgical anesthesia by using the isoflurane gas anesthesia assembly. Hairs were clipped by use of hair clipper (Roboz Surgicals, USA) and skin was wiped with the help of 70% alcohol. For brain, head and neck (oral cavity) and bone and soft tissues (muscle) tumors, small skin incision was made in the back region at lumbar vertebra level. Small pocket was created under the skin below the paralumbar fossa by inserting the pointed scissor by blunt dissection in closed position and opening forward. Tumor pieces of brain, head and neck (oral cavity) and bone and soft tissues (muscle) were slipped from the opening in the skin and retained below the paralumbar fossa as per the standard protocol (Morton and Houghton, 2007 and Frapolli et al., 2010). Skin wound was sealed with the help of surgical glue, Vetbond (3M, USA). For brain tumor tissues, 4 different tumor samples grown in mice were implanted in 36 mice. For oral cavity tumor tissues, 2 different tumor samples grown in mice were implanted in 20 mice. For muscle tumor tissues, 2 different tumor samples grown in mice were implanted in 11 mice.

For breast tumor tissues, small skin incision was made at the level of paralumbar fossa and small pocket was made with the help of pointed scissor near the mammary fat pad in a similar fashion as above. Breast tumor pieces were slipped from the
opening in the skin and retained orthotopically in the vicinity of the mammary tissue. Skin wound was sealed with the help of surgical glue, Vetbond (3M, USA). For breast tumor tissues, 3 different tumor samples grown in mice were implanted in 49 mice.

Time taken to achieve 15 mm diameter of tumor size in these mice was also recorded. Animals were sacrificed by CO₂ inhalation and small piece of tumor as well as metastasis suspected lung and liver tissues were excised aseptically and snap frozen for DNA extraction. Piece of tumor was frozen in liquid nitrogen using screw cap SV₂ vials (Tarsons, Kolkata, India) containing freezing media as above. Small piece of tumor tissue and vital organs like lungs, liver, kidney, spleen and brain was fixed in buffered neutral formalin for histopathology studies.

3.7. Revival of frozen tumor samples.

Sixteen different frozen brain tumor samples, 11 breast tumor samples, 9 oral cavity tumor samples and 6 muscle tumor samples in SV₂ vials were taken out from the liquid nitrogen and directly dipped in 37°C water for 60-90 seconds. Tumor pieces were removed from the freezing media and were washed 3 times in fresh RPMI 1640 medium. Single tumor piece of approx. 5x5 mm size was inoculated subcutaneously/orthotopically in fresh SCID mice as above in equal number of mice. Small piece of revived tumor tissue was fixed in buffered neutral formalin for histopathology studies. Time taken to achieve 15 mm diameter tumor size in SCID mice was measured. Animals were sacrificed by CO₂ inhalation. Histopathological studies of the vital organs were conducted to see the metastasis, if any.

3.8. Processing of tissue for H & E staining.

For brain tumor samples, vital tissues from 10 primary tumor grown mice and 27 serially transplanted tumor grown mice were collected in buffered neutral formalin for histopathology. For oral cavity tumor samples, vital tissues from 9 primary tumor grown mice and 13 serially transplanted tumor grown mice were collected in buffered neutral formalin for histopathology. For muscle tumor samples, vital tissues from 11 primary tumor grown mice and 6 serially transplanted tumor grown mice were collected in buffered neutral formalin for histopathology. For breast tumor samples, vital tissues from 23 primary tumor grown mice and 38 serially transplanted tumor grown mice were collected in buffered neutral formalin for histopathology.
After the samples were fixed for at least 24 hrs, tissue samples were subjected to routine H & E staining (Coolidge and Howard, 1979). In short tissues were washed overnight under running water to remove the fixative from the tissue before processing. The washed tissue samples were subjected to dehydration by increasing concentration of alcohol starting from 70%, 80%, 95% and finally two changes of absolute alcohol using automatic tissue processor (Shandon Southern, Cheshire, England; model- Duplex Processor). The tissues were then dipped in xylene twice for half an hour to make them clear and transparent. The tissues were then immersed in equal part of 60°C molten paraffin and xylene solution for half an hour to infiltrate the molten paraffin into the tissues. The tissues then transferred to fresh molten paraffin for two hours and two such changes were given to complete the infiltration process where paraffin molten wax completely replaces the xylene. These tissues were then placed in the molten paraffin in the mould as per the required orientation. The cassette was placed on the mould and molten paraffin is again poured onto the tissue and allowed to cool and harden. The hardened tissue blocks were fixed onto the microtome (Leica Microsystems, Nussloch, Germany; model- RM 2145) and sectioned into thin sections of 5-6 micrometer using a sharp microtome blade to form a thin ribbon of paraffin sections. These ribbons were then placed on a water bath having temperature of 50-55°C. Once spread, they are collected on a duly numbered egg albumin coated glass slide for staining. The sections were left at room temperature for drying and removal of water.

The sections were de-waxed in xylene for 15 min. by giving two changes each. The slides were touched on paper towel to remove the excess xylene from the slides. The slides were then dipped in 90% and 70% alcohol for 15 min each. The slides were then dipped in absolute alcohol for 15 min each.

The slides were then dipped in hematoxylin for 2-3 min and washed under running tap water for 10 min and placed them in distilled water for 5 min. The sections were then differentiated by single dipping in 1% acid alcohol and by giving a wash under tap water. The slides were then rinse in 70% and 90% alcohol for 15 min each. The sections were then counterstained by eosin solution for 2-3 min. The sections were then counterstained by eosin solution for 2-3 min. The sections were then dipped in absolute alcohol for two times. The sections were then counterstained by eosin solution for 2-3 min. The sections were dipped in xylene for 2 hrs.
to make them clear of the paraffin, if any. The sections were then mounted by the cover slip with the help of DPX mountant (Merck Chemicals, Mumbai, India; product no.- 61803502501730). The slides were dried in 37°C incubator (Centrofix, Mumbai, India) for at least 3 hrs. After drying, the slides were read under light microscope (Nikon Instruments Inc., Melville, Japan; model- Eclipse E200)

3.9. Processing of tissue for diagnosis of human tumor by PCR method.

3.9.1. DNA extraction and quantitation.

Tumor tissues grown in the SCID mice, tail samples, metastasis suspected lung and liver tissues were snap frozen in liquid nitrogen and then stored in -80 refrigerator till extraction of DNA. DNA’s were extracted from the tail and tumor tissues as well as lung and liver samples using routine method of phenol chloroform extraction and ethanol precipitation as described by Melo et al., 2006. In short tail, tumor tissues, lungs and liver tissues were transferred to sterile 1.5 ml capacity Eppendorff tubes. Tissues were digested overnight in 300 µl digestion buffer containing 18 µl of proteinase-K (20 mg/ml in 1X TE Buffer) at 50°C in a roller mixer incubator (Trishul Equipments, Mumbai, India).

Next day 200 µl of digestion buffer was added to the digestion mixer and mixed thoroughly. Equal volume of Tris-saturated phenol was added and mixed gently by inverting the tubes for at least 10 min on a rocker (Trishul Equipments, Mumbai, India). The tubes were spun down at 8000 rpm for 10 min using a cold centrifuge at 20°C (Plasto Crafts, Mumbai, India, model- Rota 4 R-V/FA). The aqueous (top) layer was transferred into a fresh Eppendorff tube. Equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) was added and mixed gently by inverting the tubes for 10 min. and spun again at 8000 rpm for 10 min. The aqueous (top) layer was transfer into a fresh Eppendorff tubes. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mix gently by inverting the tubes for 10 min. and spun at 10000 rpm for 10 min. The aqueous (top) layer was transferred into a fresh Eppendorff tube. Equal volume of chloroform was added and mix gently by inverting the tubes for 10 min. and spun at 10000 rpm for 10 min. The aqueous (top) layer was transferred into a fresh Eppendorff tube. One tenth volume of 3M sodium acetate was added to the aqueous layer (i.e. if the aqueous layer is 500 µl then 50 µl was added). Then 7/10th volume of iso-propanol was added to the
aqueous layer (i.e. if total aqueous layer is 500 µl then 350 µl of iso-propanol was added), mixed gently to allow DNA to clump, and incubated at room temperature for 10 min. It was spun at 10000 rpm for 10 min. Supernatant was discarded and 500 µl of 70% ethanol was added onto the pellet. It was incubated for 10 min at room temperature and spun down at 10000 rpm for 10 min. The supernatant was discarded and the pellet was allowed to dry at room temperature or at 37°C. The pellet was re-suspended into 50-100 µl of TE buffer and RNase (pH 8.0) and incubated at 37°C for one hour or till the pellet is dissolved completely. Required quantity of DNA was aliquoted in Eppendroff tubes, labeled properly and stored in a freezer at -20°C until use. Quantification of DNA's extracted by this method was measured with the help of Nanodrop software.

3.9.2. **PCR method for diagnosis of human tumor origin or metastasis.**

DNA's extracted by above method were subjected for the PCR amplification using human specific microsatellite primers (Ruitberg et al., 2001; Butler, 2006 and Butler, 2007; [http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi?uid=71886](http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi?uid=71886), Annexure 2). PCR was performed using human specific microsatellite primer sequences (forward primers 5'-TGCCATTTGTGGGTACATTC-3' and reverse primer 5'-TTGTGTTTCTTGTCTTACCTACA-3') which generate a 275 bp amplification product. In short 50-100 ng of target DNA was amplified in 12.5 µl reaction volume containing 0.5 U of TaqDNA polymerase (Invitrogen, USA), 0.2 µM each oligonucleotide primers (Sigma Aldrich Chemicals Pvt. Ltd, Bangalore, India), 10 mM Tris buffer (Sigma Chemicals, USA), 2.5 mM MgCl₂, 0.2 mM of each dNTP (Fermentas, USA) and sterile MilliQ water to make up the volume of the reaction to 12.5 µl. PCR consisted of 5 m of denaturation at 94°C followed by 34 cycles of 20 s denaturation at 94°C, 20 s annealing at 58°C, and 3 min of elongation at 72°C in a thermal cycler (Eppendorf, India). Labeled PCR product (15 µl) were electrophoretically separated using 2% molecular biology grade agarose gel (SRL, Mumbai, cat. no. 0144162 or Ambresco, USA, cat. no. N605) in a electrophoresis unit (Advance Co. Ltd., Tokyo, Japan; model- Mupid-2plus), visualized by UV light and photographed using gel documentation system (Make). Standard DNA marker (Fermentas, USA; cat. no. SM0241) of 100 bp size was run every time for easy determination of the size of the reaction product developed after electrophoresis.

3.10.1. Brain tumor response.

Small Tumor tissue pieces of 5x5 mm blocks of brain tumor were implanted aseptically in 10 SCID mice as above. Tumor growth was measured with the help of vernier caliper. Control group animals were maintained without any treatment. Treatment group animals were injected with Paclitaxel (Dr. Reddy’s Laboratories, Hyderabad, India; Trade name- Mitotax) at the rate of 25 mg/kg body weight of the animals diluted four times with the normal saline. Paclitaxel was injected on 1, 5, and 9th day starting after the tumor reached 50-300 mm³ size which occurred on 5-7th day after transplant (Nakayama et al., 2009). Subsequently, tumor size was measured every week for next 12-20 weeks with the help of electronic vernier caliper. Tumor volume was calculated in cubic mm using the formula (W²xL)/2, where W= tumor width and L= tumor length in mm (Nemati et al., 2010). Data was assessed using the line graph. Anticancer drug effect was assessed as ineffective when the tumor started growing beyond 500 or 1000 mm³ (Teicher, 2006).

3.10.2. Breast tumor response.

Small Tumor tissue pieces of 5x5 mm blocks of breast tumor were implanted aseptically in 19 SCID mice as above. Tumor growth was measured with the help of vernier caliper. Control group animals were maintained without any treatment. Treatment group animals were injected with Paclitaxel at the rate of 25 mg/kg body weight of the animals diluted four times with the normal saline. Paclitaxel was injected on 1, 5, and 9th day starting after the tumor reached 50-300 mm³ size which occurred on 5-7th day after transplant (Nakayama et al., 2009). Subsequently, tumor size was measured every week for next 12-20 weeks with the help of electronic vernier caliper. Tumor volume was calculated in cubic mm using the formula (W²xL)/2, where W= tumor width and L= tumor length in mm (Jensen et al., 2008). Data was assessed using the line graph. Anticancer drug effect was assessed as ineffective when the tumor started growing beyond 500 or 1000 mm³ (Teicher, 2006).

3.10.3. Oral cavity tumor response.

Small Tumor tissue pieces of 5x5 mm blocks of oral cavity tumor were implanted aseptically in 13 SCID mice as above. Tumor growth was measured with the help of vernier caliper. Control group animals were maintained without any treatment.
Treatment group animals were injected with Paclitaxel at the rate of 25 mg/kg body weight of the animals diluted four times with the normal saline. Paclitaxel was injected on 1, 5, and 9\textsuperscript{th} day starting after the tumor reached 50-300 mm\textsuperscript{3} size which occurred on 5-7\textsuperscript{th} day after transplant (Nakayama et al., 2009). Subsequently, tumor size was measured every week for next 12-20 weeks for control as well as treatment group with the help of electronic vernier caliper. Tumor volume was calculated in cubic mm using the formula \( \frac{W^2 \times L}{2} \), where \( W \) = tumor width and \( L \) = tumor length in mm (Jensen et al., 2008). Data was assessed using the line graph. Anticancer drug effect was assessed as ineffective when the tumor started growing beyond 500 or 1000 mm\textsuperscript{3} (Teicher, 2006).

3.11. Statistical analysis.

Data of differences between response of brain, breast and oral cavity tumor to the standard anticancer drug, Paclitaxel was analyzed for significance using student's 't' test. A probability value was deemed statistically significant if \( p < 0.05 \).